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(54) Title: OINTMENTS

(54) Title of Invention: Ointments

(57) Abstract: Ointments containing chemically modified physiologically active polypeptides (wherein the chemically modified physiologically active polypeptides are exemplified by physiologically active polypeptides chemically modified with at least one polyalkylene glycol and the physiologically active polypeptides to be chemically modified are exemplified by superoxide dismutase *[sp]*, interferon-α, interferon-β, interferon-γ and granulocyte colony-simulating factor).

(57) Abstract:

Ointments containing chemically modified physiologically active polypeptides (wherein the chemically modified physiologically active polypeptides are exemplified by physiologically active polypeptides chemically modified with at least one polyalkylene glycol and the physiologically active polypeptides to be chemically modified are exemplified by superoxide dismutase, interferon-α, interferon-β, interferon-γ, and granulocyte colony-simulating factor).

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Specification
Ointments

Technical Field

The objective of this invention relates to ointments that contain chemically modified physiologically active polypeptides. Furthermore, it relates to the application of said ointments as therapeutic, cosmetic, and moisturizing agents.

Background Art

There are many cases wherein there is instability in polypeptides that possess physiological activity (hereafter called physiologically active polypeptides) and are used as a therapeutic agent for a specific disease when administered into blood and where a sufficient pharmacological effect cannot be expected. For example, physiologically active polypeptides having a molecular weight of less than 60,000 administered into blood are mostly excreted into urine by renal glomerular filtration, and their use as therapeutic agents is not expected to give a significant therapeutic effect and often requires repeated administration. In addition, other physiologically active polypeptides are degraded by hydrolases and the like present in the blood stream thereby losing their physiological activities.

Some exogenous physiologically active polypeptides have physiological activities effective for the treatment of diseases, but it is known that such exogenous physiologically active polypeptides and physiologically active polypeptides produced by recombinant DNA techniques sometimes induce immunoreaction when administered into blood to cause serious side-effects such as anaphylactic shock owing to the difference in structure between them and endogenous physiologically active polypeptides. In addition, some physiologically active polypeptides have physical properties unsuitable for use as therapeutic agents, e.g. poor solubility.

One attempt to solve these problems when using physiologically active polypeptides as therapeutic agents is use of a method known to chemically bind at least one molecule of an inactive polymer chain to physiologically active polypeptides. In many cases, desired characteristics are invested on the polypeptides or proteins by chemically binding polyethylene glycols such as polyalkylene glycol group to said polypeptides.

For example, superoxide dismutase (SOD) modified with polyethylene glycol has a remarkably prolonged half-life in the bloodstream and demonstrates a durable action as found in Pharmaceutical Research Communication (Pharm. Research Commun.), Vol. 19, p. 287 (1987). There is also a report of modification of granulocyte colony-stimulating factor (G-CSF) with polyethylene glycol as found in The Journal of Biochemistry (J. Biochem.), Vol. 115, p. 814 (1994). Gillian E. Francis, et al. summarized examples of polyethylene glycol-modified polypeptides such as asparaginase, glutaminase, adenosine deaminase and uricase in Pharmaceutical Biotechnology, Vol. 3, "Stability of Protein Pharmaceuticals, Part B", p. 235 (1992), Plenum Press New York (Plenum Press, New York).

Further, it is known that modification of physiologically active polypeptides with polyalkylene glycols produce effects such as enhancement of thermal stability [*Seibutsubutsuri* (Biophysics), Vol. 38, p. 208 (1998)] and solubilization in organic solvents [Biochemical and Biophysical Research Communications, B.B.R.C.)] Vol. 122, P. 845 (1984).

From a different perspective, methods are also known for binding polyalkylene glycols to peptides or proteins, by introducing an active ester of carboxylic acid, a maleimide group, a carbonate group, cyanuric chloride, a aldehyde group, an epoxide group or the like to an end of a polyalkylene glycol and binding it to an amino group or a thiol group in a polypeptide [Bioconjugate Chem., Vol. 6, p. 150 (1995)]. These techniques include the binding of a polyethylene glycol to a specific amino acid residue in a physiologically active polypeptide, which causes enhancement of stability in blood without impairing the biological activities of the peptide or protein. Examples of the polyethylene glycol modification specific to amino acid residues in physiologically active polypeptides include the binding of a polyethylene glycol to the carboxyl terminus of a growth hormone-releasing factor through norleucine as a spacer [J. Peptide Res., Vol. 49, p. 527 (1997)] and the specific binding of a polyethylene glycol to cysteine introduced to the 3-position of interleukin-2 by recombinant DNA techniques [Bio/Technology, Vol. 8, p. 343 (1990)].

With the above physiologically active polypeptides modified by polyalkylene glycol, it is generally known that improvements in safety and sustaining blood levels by means of intramuscular and subcutaneous administration have been achieved however, whether or not physiologically active polypeptides modified by polyalkylene glycol can be administered in other than an injectable form has not been sufficiently resolved.

On the other hand, ointments, eye drops, nasal drops, respiratory drops, ear drops, suppositories, creams, lotions, liniments, vapor agents, and bandages are known local therapeutic agents. For instance, burn therapy using Epidermal Growth Factor (EGF) and SOD exemplifying utilization of an ointment derived from a physiologically active polypeptide is reported in the Biological and Pharmaceutical Bulletin Vol. 16 (11), p. 1146 (1993).

In addition, interferon as a local therapeutic agent in an ointment has been tested clinically. That is, interferon ointment usability with viral dermatoses such as herpes zoster, herpes labialis, corneal herpes, communicable papilloma, varicella, and stomatitis, and with opthalmopathy such as macular degeneration, diabetic retinitis, central retinal vein occlusion, and glaucoma is reported. Specifically, interferon-α is known as a therapy for herpes simplex virus type I [Dermatology , Vol.184 (I), p. 40 (1992), Acta Virologica, Vol. 39 (3), p. 125 (1995)] and leukocytic interferon therapy is known for ulcers [International Journal of Clinical Pharmacology, Therapy and Toxicology, Vol. 19 (10), p. 450 (1981). In addition, the effect of an interferon ointment as a therapeutic agent for visual disorders with specific angiogenesis is reported in [Kokai patent H09-255555, [*Nihon Ganka Gakkai Zasshi* (Japan Ophthalmic Society Journal) Vol. 99, p. 571 (1995)].

Although immediate focal area results were obtained with these therapeutic methods and remarkable therapeutic results were observed, no conclusion of stability of these local therapeutic agents that contain physiologically active polypeptides was reached.

By modifying physiologically active polypeptides with a polyalkylene glycol, we observed consistent experimental results of a stable physiologically active polypeptide for use as an active ingredient in an ointment, and a physiologically active polypeptide that sustained exceptional stability and high physiological activity.

Invention Disclosure

The objective of this invention is to provide ointments that contain physiologically active polypeptide derivatives with superior stability in ointments compared to a physiologically active polypeptide.

In accomplishing the objective, the inventors observed that when a chemical modifier, for instance a polyalkylene glycol, modified a physiologically active polypeptide exemplified by an interferon, said physiological activity was sustained and stability within the ointment maintained in comparison with an unmodified physiologically active polypeptide, ultimately completing this invention.

That is, this invention relates to ointments containing physiologically active polypeptides that have been chemically modified (hereafter described as chemically modified physiologically active polypeptides).

In addition, this invention relates to ointments used for cosmetic purposes and for moisture retention containing the aforementioned chemically modified physiologically active polypeptides.

Furthermore, this invention relates to application of chemically modified physiologically active polypeptides used to manufacture ointments, cosmetic ointments, and moisture-retention ointments.

In addition, this invention relates to methods of stabilizing ointments with physiologically active polypeptides characterized by modification of physiologically active peptides with a polyalkylene glycol. Furthermore, this invention relates to methods that sustain activity with said physiologically active polypeptides characterized by modification of physiologically active polypeptides with a polyalkylene glycol.

In addition, this invention relates to compositions containing chemically modified physiologically active polypeptides in an ointment base.

The following explains this invention in detail.

With respect to chemically modified physiologically active polypeptides, a chemically modified physiologically active polypeptide with, for instance, at least one polyalkylene glycol is demonstrated.

However, the preferred polypeptide from among physiologically active polypeptides is one that contains physiological and pharmacological activity. For example, the following can be cited: asparaginase, glutaminase, arginase, uricase, superoxide dismutase, lactoferin, streptokinase, plasmin, adenosine deaminase, interleukin 1～18, interferon-*α* interferon-β, interferon-γ, interferon-ω, interferon-τ, granulocyte-colony stimulating factor, erythropoietin, tumor necrosis factor, thrombopoietin, Klotho protein, leptin, fibroblast growth factor 1～19, midkine, calcitonin, epidermal growth factor, glucagon, insulin-like growth factor 1, osteogenic protein 1, stem cell factor, amylin, parathyroid hormone, plasminogen activator, vascular endothelial growth factor, transforming growth factor, glucagon-like peptide, natriuretic peptide, plasminogen, angiopoietin, angiostatin, endostatin, hepatocyte growth factor, antibodies or antibody fragments, polypeptides having physiological activity from hybrid antibodies and their amino acid complexes, amino acid deletion variant, carbohydrate addition, carbohydrate deletion variant, and partial peptides.

More preferred physiologically active polypeptides are physiologically active peptides having interferons such as interferon-β, interferon-α, and interferon–γ, and superoxide dismutase activity.

These physiologically active polypeptides can be obtained not only by extraction from animal organs and tissues, but also by ordinary peptide synthesis and recombinant DNA techniques. Furthermore, commercially available physiologically active polypeptides may be used. The physiologically active polypeptide used in the chemical modification may be a partially purified product or a product purified to purity suitable for chemical modification by purification methods such as gel filtration chromatography, ion-exchange chromatography, hydrophobic chromatography, reversed phase chromatography and extraction.

The physiologically active polypeptide is produced in a buffer such as a phosphate buffer, a borate buffer, an acetate buffer or a citrate buffer, water, an appropriate organic solvent such as N,N-dimethylformamide, dimethyl sulfoxide, dioxane or tetrahydrofuran, or a mixed solvent of such an organic solvent and an aqueous solution, and then used in the chemical modification reaction.

With the condition that it be capable of achieving the objective of this invention, a chemically modified physiologically active polypeptide or any form thereof is acceptable; for example, a physiologically active polypeptide modified with a polyalkylene glycol such as polyethylene glycol or a derivative thereof, polypropylene glycol or a derivative or a polyethylene glycol-polypropylene glycol copolymer or derivative is preferred. A polyalkylene glycol with a straight chain structure or one with a branched chain structure with two or more branches may be used; the average molecular weight of the polyalkylene glycol of approximately 1,000 to 1,000,000 is acceptable but an average molecular weight of 5,000 to 100,000 is preferred.

The polyalkylene glycol may be a commercially available product or may be prepared using previously known methods.

The method expressed by Samuel Zalipsky, et al. [Bioconjugate Chem., Vol. 6, p. 150 (1995)] is a polyalkylene glycol preparation method and can be used to prepare the same.

The reaction between the physiologically active polypeptide and the polyalkylene glycol is produced by a reaction using a branched polyalkylene glycol between 1～1000 mols of polyalkylene glycol per mol of physiologically active polypeptide, but a ratio of 1～50 moles per mol of a physiologically active polypeptide is preferred. The degree of modification of the physiologically active polypeptide with the branched polyalkylene glycol can be arbitrarily selected by controlling the molar ratio of the branched polyalkylene glycol to the physiologically active polypeptide, reaction temperature, pH, reaction time, etc. The solvent used in the reaction may be any of the solvents that do not interfere with the reaction, for example, a phosphate buffer, a borate buffer, a tris-hydrochloride buffer, an aqueous sodium hydrogencarbonate solution, a sodium acetate buffer, N,N-dimethylformamide, dimethyl sulfoxide, methanol, acetonitrile or a dioxane. The reaction temperature, reaction time, pH and other factors are not limited so long as the activity of the physiologically active polypeptide is not impaired under the conditions. For example, the reaction is preferably carried out at 0～50° C and at a pH of 4 to 10 for 10 minutes～100 hours.

The physiologically active polypeptide modified with the branched polyalkylene glycol can be purified by gel filtration, ion-exchange chromatography, reverse phase high performance liquid chromatography, affinity chromatography, ultrafiltration or the like in a usual manner. Confirmation of the polypeptide structure in the synthesized or purified physiologically active polypeptide or the physiologically active polypeptide modified with the branched polyalkylene glycol can be carried out by mass spectrometry, nuclear magnetic resonance (NMR) and amino acid composition analysis using an amino acid analyzer, and also by amino acid sequence analysis using a gas phase protein sequencer in which phenylthiohydantoin (PTH) amino acid obtained by Edman degradation is analyzed by reverse phase HPLC.

The branched polyalkylene glycols can also be used for site-specific covalent modification of polypeptides, more specifically and preferably, all natural or recombinant polypeptides having a free cysteine residue such as granulocyte-colony stimulating factor (G-CSF), erythropoietin, interferons and interleukins.

In one preferred form of this invention, a chemically modified physiologically active polypeptide is the polyethylene glycol modified physiologically active polypeptide described in Japanese Kokai Patent H11-310600, the polyethylene glycol modified physiologically active polypeptide described in WO99/55377, the polyethylene glycol modified physiologically active polypeptide described in WO00/23114, the polyethylene glycol modified physiologically active polypeptide described in Japanese Tokuhyo Patent S62-503171, WO87/00056, US4766106, US4917888, US4863727, or the polyethylene glycol modified physiologically active polypeptide described in Japanese Kokai H6-192300 and EP593868A1.

Another preferred form of this invention involves branched polyalkylene glycols wherein a polyalkylene glycol that modifies a physiologically active polypeptide, two single-chain polyalkylene glycols and one group having reactivity with an amino acid side chain, the N-terminal amino group or the C-terminal carboxyl group in a polypeptide or one group convertible into the group having reactivity are bound.

Among polyalkylene glycols that modify a physiologically active polypeptide, preferred branched polyalkylene glycols are compounds represented by Equation (I):

$(R^1\text{-}M_n\text{-}X^1)_2L(X^2\text{-}X^3\text{-}R^2)_q$ (I)

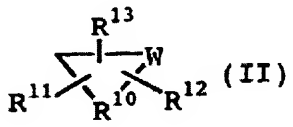
{wherein L represents groups which can have between 3 and 5 branches including a cyclic structure other than a planar structure; M represents OCH_2CH_2, $OCH_2CH_2CH_2$, $OCH(CH_3)CH_2$, $(OCH_2CH_2)_r - (OCH_2CH_2CH_2)_s$ (in which r and s, which may be the same or different, each represent an arbitrary positive integer) or $(OCH_2CH_2)_{ra} - [OCH(CH_3)CH_2)_{sa}$ (in which ra and sa have the same meanings as the above r and s, respectively);
n represents an arbitrary positive integer;
q represents an integer of 1 to 3;

R^1 represents a hydrogen atom, lower alkyl or lower alkanoyl;
R^2 represents a group having reactivity with an amino acid side chain, an N-terminal amino group or a C-terminal carboxyl group in a polypeptide or a group convertible into a group having this reactivity;
X^1 represents a bond, O, S, alkylene, $(OCH_2)_{ta}$ (in which ta represents an integer of 1 to 8), $(CH_2)_{tb}O$ (in which tb has the same meaning as the above ta), NR^3 (in which R^3 represents a hydrogen atom or lower alkyl), R^4-NH-C(=O)-R^5 [in which R^4 represents a bond, alkylene or $O(CH_2)_{tc}$ (in which tc has the same meaning as the above ta) and R^5 represents a bond, alkylene or OR^{5a} (in which R^{5a} represents a bond or alkylene)], R^6-C(=O)-NH-R^7 [in which R^6 represents a bond, alkylene or $R^{6a}O$ (in which R^{6a} has the same meaning as the above R^{5a}) and R^7 represents a bond, alkylene or $(CH_2)_{td}O$ (in which td has the same meaning as the above ta)], R^8-C(=O)-O (in which R^8 has the same meaning as the above R^{5a}) or O-C(=O)-R^9 (in which R^9 has the same meaning as the above R^{5a});
X^2 represents a bond, 0 or $(CH_2)_{te}O$ (in which te has the same meaning as the above ta);
X^3 represents a bond or an alkylene; and groups of R^1-M_n-X^1 and between one and three groups of identical or different X^2-X^3-R^2}.

In formula (I), a branched polyalkylene glycol where q equals 1 is preferred, and a branched polyalkylene glycol where n is between 10 and100,000, and where r and s, and ra and sa are either the same or different value of between 1 and100,000 is also preferred.

In formula (I), preferred branched polyalkylene glycols are where R^2 represents a hydroxyl group, carboxy, formyl, amino, vinylsulfonyl, mercapto, cyano, carbamoyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, oxiranyl, lower alkanoyloxy, maleimide, succinimidooxycarbonyl, substituted or unsubstituted aryloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidooxycarbonyl, imidazolylcarbonyl, substituted or unsubstituted lower alkoxycarbonyloxy, substituted or unsubstituted aryloxycarbonyloxy, tresyl, lower alkanoyloxycarbonyl, substituted or unsubstituted aroyloxycarbonyl, substituted or unsubstituted aryldisulfide, or azide.

In formula (I), the preferred L represents a group formed by removing between 3 and 5 hydrogen atoms from a compound represented as formula (II)



(II)

[wherein R^{10} represents $(CH_2)_u$ (in the formula, u represents an integer between 1 and 10) or $CH=CH\text{-}(CH_2)_{ua}$ (in the formula, ua represents an integer between 1 and 8),
R^{11}, R^{12}, and R^{13}, which may be the same or different, represent a hydrogen atom, hydroxyl group, substituted or unsubstituted lower alkyl, lower alkoxy, amino, carboxy, cyano, formyl or the like,
W represents O, S, CH_2, or NR^{14} (in the formula, R^{14} represents a hydrogen atom or a lower alkyl). Of these, branched polyalkylene glycols where W is CH_2 and u is 4 are preferred.

Yet another preferred form of this invention is that the polyalkylene glycol that modifies a physiologically active polypeptide be a branched polyalkylene glycol capable of binding to a group convertible into a group having reactivity with an amino acid side chain, the N-terminal amino group or the C-terminal carboxyl group in a polypeptide and is capable of having as branches three or more molecules of single-chain polyalkylene glycols.

Among polyalkylene glycols that modify a physiologically active polypeptide,

$$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1x})_{mx}Lx(X^{2s}\text{-}X^{3x}\text{-}R^{2x})_{qx} \qquad (Ix)$$

formula (Ix) is the branched polyalkylene glycol expressed, wherein [in the formula, Lx represents a group capable of having four or more branches, Mx represents OCH_2CH_2, $OCH_2CH_2CH_2$, $OCH(CH_3)CH_2$, $(OCH_2CH_2)_{rx}$-$(OCH_2CH_2CH_2)_{sx}$ (in the formula, rx and sx, which may be the same or different, represent any arbitrary integer) or OCH_2CH_{2rax}-$[OCH(CH_3)CH_2]_{sax}$ (in the formula, rax and sax are identical to the aforementioned rx and sx, respectively),

nx represents any arbitrary integer, mx represents any integer ≥3, qx represents an integer between 1 and 3, R^{1x} represents a hydrogen atom, lower alkyl, or lower alkanoyl,
R^{2x} represents groups capable of binding to a group convertible into a group having reactivity with an amino acid side chain, the N-terminal amino group or the C-terminal carboxyl group in a polypeptide, X^{1x} represents a bond, O, S, alkylene, $(OCH_2)_{tax}$ (in the formula, tax represents an integer of 1 to 8), $(CH_2)_{tbx}O$ (in the formula, tbx has the same meaning as the above tax), NR^{3x} (in the formula, R^{3x} represents a hydrogen atom or lower alkyl), R^{4x}-NH-C(=O)-R^{5x} [in the formula, R^{4x} represents a bond, alkylene or $O(CH_2)_{tcx}$ (in the formula, tcx has the same meaning as the above tax), R^{5x} represents a bond, alkylene or OR^{5ax} (in the formula, R^{5ax} represents a bond or alkylene)], R^{6x}-C(=O)-NH-R^{7x} [in the formula, R^{6x} represents a bond, alkylene or $R^{6ax}O$ (in the formula, R^{6ax} has the same meaning as the above R^{5ax}), R^{7x} represents a bond, alkylene or $(CH_2)_{tdx}O$ (in the formula, tdx has the same meaning as the above tax)], R^{8x}-C(=O)-O (in the formula, R^{8x} has the same meaning as the above R^{5ax}) or O-C(=O)-R^{9x} (in the formula, R^{9x} has the same meaning as the above R^{5ax});
X^{2x} represents a bond, O or $(CH_2)_{tex}O$ (in the formula, tex has the same meaning as the above tax);
X^{3x} represents a bond or alkylene;
and three or more R^{1x}-$(Mx)^{nx}$-X^{1x} may each be the same or different, and when two or three X^{2x}-X^{3x}-R^{2x} are present (when qx is 2 or 3) they may be the same or different}.

In formula (Ix), qx is equal to 1 for the preferred branched polyalkylene glycol, and mx is equal to 3 or 4 for the preferred branched polyalkylene glycol. Furthermore, in the preferred branched polyalkylene glycol, nx is between 10 and 100,000, rx and sx, and rax and sax, may be the same or different, and are between 1 and 100,000.

In formula (Ix), the R^{2x} in the preferred branched polyalkylene glycol may be a hydroxyl group, carboxy, formyl, amino, vinylsulfonyl, mercapto, cyano, carbamoyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, oxiranyl,

lower alkanoyloxy, maleimide, succinimidooxycarbonyl, substituted or unsubstituted aryloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidooxycarbonyl, imidazolylcarbonyl, substituted or unsubstituted lower alkoxycarbonyloxy, substituted or unsubstituted aryloxycarbonyloxy, tresyl, lower alkanoyloxycarbonyl, substituted or unsubstituted aroyloxycarbonyl, substituted or unsubstituted aryldisulfide, or azide.

In addition, among the branched polyalkylene glycols stated above, a branched polyalkylene glycol with a molecular weight of between 500 and 1,000,000 is preferred.

In formula (Ix), Lx in the preferred branched polyalkylene glycol may be a group selected from among a group formed by removing four or more hydrogen atoms from tricine, a group formed by removing four or more hydrogen atoms from shikimic acid, a group formed by removing four or more hydrogen atoms from quinic acid, a group formed by removing four or more hydrogen atoms from erythritol, a group formed by removing four or more hydrogen atoms from pentaerythritol, and a group formed by removing four or more hydrogen atoms from glucose.

Hereafter, the compounds represented by formula (I) and formula (Ix) will be referred to as Compounds (I) and Compounds (Ix), respectively. The same shall apply to the compounds of other formula numbers.

In the definitions of the groups in formula (I), the lower alkyl and the lower alkyl moiety of the lower alkanoyl include linear or branched alkyl groups having 1 to 8 carbon atoms such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, pentyl, neopentyl, hexyl, heptyl and octyl. The alkylene includes alkylene groups having 1 to 8 carbon atoms such as methylene, ethylene, n-propylene, isopropylene, n-butylene, isobutylene, sec-butylene, tert-butylene, pentylene, neopentylene, hexylene, heptylene and octylene.

In formula (I), M represents OCH_2CH_2, $OCH_2CH_2CH_2$, $OCH(CH_3)CH_2$, $(OCH_2CH_2)_r$-$(OCH_2CH_2CH_2)_s$ (in the formula, r and s, which may be the same or different, represent an arbitrary positive integer) or $(OCH_2CH_2)_{ra}$-$[OCH(CH_3)CH_2]_{sa}$ (in the formula, ra and sa have the same meanings as the above r and s, respectively), and when $(OCH_2CH_2)_r$-$(OCH_2CH_2CH_2)_s$ (in the formula, r and s each have the same meanings as defined above) or $(OCH_2CH_2)_{ra}$-$[OCH(CH_3)CH_2]_{sa}$ (in the formula, ra and sa have the same meanings as defined above), r and s, and ra and sa are preferably between 1 and 100,000 , but even more preferably between 1 and 1000.

In formula (I), the average molecular weight of the polyalkylene glycol moiety represented by M_n of approximately 1,000 to 1,000,000 is preferred, but 5,000 to 100,000 is even more preferable. When Mn is $-(OCH_2CH_2)_n-$, it is preferred that polyethylene glycols used as raw materials are monodisperse and their molecular weight distribution represented by Mw (weight average molecular weight)/Mn (numeric average molecular weight) is 1.1 or less, and commercially available products can be utilized if the average molecular weight is 30,000 or less. For example, monomethoxypolyethylene glycols having an average molecular weight of 2,000, 5,000, 10,000, 12,000, 20,000 or the like can be used.

As disclosed in formula (I), the preferred branched polyalkylene glycol is represented by formula (I) in which two single-chain polyalkylene glycols bond to a group containing a cyclic structure other than a planar structure, and the branched polyalkylene glycol is capable of binding to a group having reactivity with an amino acid side chain, the N-terminal amino group or the C-terminal carboxyl group in a polypeptide or a group convertible into a group having this reactivity, and has a molecular weight preferably in the range of 500 to 1,000,000.

In formula (I), q represents an integer of 1 to 3 and is preferably 1.

In formula (I), L represents a group capable of having between 3 and 5 branches that contains a cyclic structure other than a planar structure and may have a hydroxyl group, substituted or unsubstituted lower alkyl, lower alkoxy, amino, carboxy, cyano, formyl or the like as a substituent group on the cyclic structure. Here, the lower alkyl and the lower alkyl moiety of the lower alkoxy have the same meaning as the above lower alkyl, and the substituent in the substituted lower alkyl includes a hydroxyl group, amino, lower alkanoyloxy, lower alkanoylamine, lower alkoxy, lower alkoxyalkoxy, lower alkanoyl, lower alkoxycarbonyl, lower alkylcarbamoyl, lower alkylcarbamoyloxy and the like. The lower alkyl moiety of the lower alkanoyloxy, the lower alkanoylamino, the lower alkoxy, the lower alkoxyalkoxy, the lower alkanoyl, the lower alkoxycarbonyl, the lower alkylcarbamoyl and the lower alkylcarbamoyloxy have the same meaning as the above lower alkyl.

Examples of L include groups formed by removing between 3 and 5 hydrogen atoms from cyclohexanes, cyclohexenes, or monosaccharides. Specific examples of said cyclohexanes, cyclohexenes, and monosaccharides are cyclohexane tricarboxylic acid, cyclohexanoltriol, 1,3,5, trimethyl-1,3,5 cyclohexane tricarboxylic acid (Kemp's triacid), quinic acid, diaminocyclohexane, 2,4,10-trioxa adamantan, inositol, shikimic acid, D,L-sorbitol, ribose, erythritol, and their stereoisomers.

The structure of the L moiety can be constructed by using a commercially available compound without modification, or using the compound through conversion into a derivative suitable for the binding of polyalkylene glycols according to a general organic synthetic method, or using the compound after the protection of a functional group [The Chemical Society of Japan, *Jikken Kagaku Koza* (Experimental Chemistry Course), 4th edition (1992), Organic Synthesis I-V, Maruzen; Protective Groups In Organic Synthesis, 2nd edition, John Wiley & Sons, Inc. (1991); etc.]

Cyclohexanes other than those mentioned above can be synthesized according to the method of Kihi, et al. [*Daiyuki Kagaku* (Great Organic Chemistry), Vol. 6, p. 183 (1958), Asakura Shoten], the method of G. E. McCasland and E. Clide Horswill [Journal of American Chemical Society, Vol. 76, p. 2373 (1954)] or the like.

In addition, the structure of the L moiety can be constructed through conversion of a compound capable of having between 3 and 5 branches containing a cyclic structure other than a planar structure by means of, for example, the method described by S. Isoda and H. Yamaguchi [Chemical and Pharmaceutical Bulletin (Chem. Pharm. Bull.), Vol. 28 (8), p. 2337 (1980)] and that described by K. Prasad and O. Repic [Tetrahedron Letters, Vol. 25 (23), p. 2435 (1984)] for compounds containing a benzene ring.

In Compounds (I), the binding of polyalkylene glycols to L through X^1 can be easily effected by combining the reactions known in the general organic synthetic methods [The Chemical Society of Japan, *Jikken Kagaku Koza* (Experimental Chemistry Course), 4th edition, pp. 19-23 (1992), Organic Synthesis I-V, Maruzen].

In formula (I), R^2 represents a group having reactivity with an amino acid side chain, the N-terminal amino group or the C-terminal carboxyl group in a polypeptide or a group convertible into a group having such reactivity.

That is, groups having the above reactivity include groups reactive with any one of the side chains of lysine, cysteine, arginine, histidine, serine, threonine, tryptophan, aspartic acid, glutamic acid, glutamine and the like, the N-terminal amino group and the C-terminal carboxyl group in a polypeptide. Examples of such groups include a hydroxyl group, carboxy, formyl, amino, vinylsulfonyl, mercapto, cyano, carbamoyl, carbonyl halide, lower alkyl halide, isocyanato, isothiocyanato, oxiranyl, lower alkanoyloxy, maleimide, succinimidooxycarbonyl, substituted or unsubstituted aryloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidooxycarbonyl, imidazolylcarbonyl, substituted or unsubstituted lower alkoxycarbonyloxy, substituted or unsubstituted aryloxycarbonyloxy, tresyl, lower alkanoyloxycarbonyl, substituted or unsubstituted aroyloxycarbonyl, substituted or unsubstituted aryldisulfide, and azide.

In the definitions of the above groups, the lower alkyl moiety of the lower alkoxycarbonyloxy, the lower alkyl halide, the lower alkanoyloxy and the lower alkanoyloxycarbonyl has the same meaning as the above lower alkyl. The aryl moiety of the aryloxycarbonyl, the aryloxycarbonyloxy and the aryldisulfide includes aryls having 6 to 14 carbon atoms such as phenyl, naphthyl, biphenyl and anthryl. The aroyl moiety of the aroyloxycarbonyl includes aroyls having 7 to 13 carbon atoms such as benzoyl, naphthoyl and phthaloyl. The halogen moiety of the carbonyl halide and the lower alkyl halide includes atoms of fluorine, chlorine, bromine and iodine each.

The substituted lower alkoxycarbonyloxy has 1 to 3 substituents which may be the same or different. Examples of the substituents are a hydroxyl group, carboxy and halogen. The halogen has the same meaning as defined above.

The substituted aryloxycarbonyl, the substituted aryloxycarbonyloxy, the substituted aryldisulfide and the substituted aroyloxycarbonyl have 1 to 3 substituents which may be the same or different. Examples of the substituents are a hydroxyl group, carboxy, halogen, cyano and lower alkyl. The halogen and the lower alkyl have the same meanings as defined above, respectively.

The group represented by R^2 may be contained in the raw material for constructing the structure of the L moiety, or may be formed by protecting a necessary functional group in the raw material compound with an appropriate protective group in advance [Protective Groups In Organic Synthesis, 2nd edition, John Wiley & Sons, Inc. (1991) etc.], removing the protective group after binding polyalkylene glycols to L through X^1 to make branches, and converting it, if necessary. Furthermore, after the polyalkylene glycols are bound to L through X^1 to make branches, the above R^2 can also be introduced to L, if necessary through X^2 or X^3, by a normal organic synthesis method.

More specifically, the branched polyalkylene glycols of the present invention can be produced, for example, by the following processes; however, the processes for producing the branched polyalkylene glycols of the present invention are not limited thereto.

Process 1: Production of compounds wherein X^1 is a bond, O, alkylene, $O(CH_2)_{ta}$ or $(CH_2)_{tb}$

Of Compounds (I), Compound (Ia), wherein X^1 is a bond, O, alkylene, $O(CH_2)_{ta}$ (in the formula, ta has the same meaning as defined above) or $(CH_2)_{tb}O$ (in the formula, tb has the same meaning as defined above) can be produced, for example, by the following process.

A polyol having two or more hydroxyl groups (hereafter, when the term cyclic polyol is used in this document, it also includes compounds containing hydroxy-lower-alkyls except when hydroxyl groups serve as substituents for cyclic structures) is dissolved or suspended in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide,

toluene, tetrahydrofuran, acetonitrile or pyridine under anhydrous conditions, and 3 mols or more of a halide or tosylate of a polyalkylene glycol or a monoalkyl ether or monocarboxylate ester thereof (hereinafter, these are collectively referred to as polyalkylene glycol A) is added thereto in the presence of 1 to 30 mols of an appropriate base (e.g. sodium hydride, zinc oxide, sodium hydroxide or triethylamine), followed by reaction at -20 to 150°C for 1 hour to 10 days to obtain a mixture containing a branched polyalkylene glycol having two chains.

The cyclic polyol is selected from commercially available compounds such as cyclohexanetriol, quinic acid, shikimic acid, glucose, sorbitol, ribose, and erythritol, or from compounds derived from these commercially available compounds. Examples of the compounds derived from the commercially available compounds include polyols obtained by reducing polycarboxylic acid selected from cyclohexane tricarboxylic acid, Kemp's triacid and the like with an appropriate reducing agent according to a normal organic synthesis method [The Chemical Society of Japan, Jikken Kagaku Koza (Experimental Chemistry Course), 4th edition, Vols. 19-21 (1992), Maruzen]. Suitable reducing agents include lithium aluminum hydride, sodium borohydride, sodium cyanoborohydride and hydrogen.

The cyclic polyol may have hydroxyl groups at any positions and can be used in the reaction after appropriate protection of a functional group unnecessary for the reaction by the method described in Protective Groups In Organic Synthesis, 2nd edition, John Wiley & Sons, Inc. (1991), etc. or conversion into a derivative.

The halide or tosylate of polyalkylene glycol A can readily be produced by various methods disclosed in a compilation by Samuel Zalipsky [Bioconjugate Chem., Vol. 6, p. 150 (1995)] and the like. The halide or tosylate of polyalkylene glycol A used for the binding may have any average molecular weight so long as the molecular weight distribution is uniform (preferably Mw/Mn is 1.1 or less).

The obtained mixture containing a branched polyalkylene glycol having two chains can be used in the next step at the purity without modification or after purifying and isolating the branched polyalkylene glycol having two chains to a desired purity by a known method such as ion-exchange chromatography, reverse phase chromatography, hydrophobic chromatography, two-phase partition or recrystallization. By the above steps, some of Compounds (Iaj), i.e. Compounds (Ia), wherein R^2 is a hydroxyl group, are obtained.

From a different perspective, the desired branched polyalkylene glycol having three or more chains can also be prepared by using a polyhalide or a polytosyl and polyalkylene glycol A. In this case, the desired compound can be obtained by dissolving or suspending between 1 and 3 molar equivalents or more of polyalkylene glycol A in an appropriate solvent, such as N,N-dimethylformamide, dimethyl sulfoxide, toluene or tetrahydrofuran, and adding 1 molar equivalent of a polyhalide or polytosyl thereto in the presence of between 1 and 30 mols of an appropriate base (e.g. sodium hydride, zinc oxide, sodium hydroxide or triethylamine) per mol of polyalkylene glycol A, followed by reaction at between -20 and 150°C for between 1 hour and 10 days.

The polyhalide may be a commercially available compound or may be obtained by converting the above polyol into a halide [The Chemical Society of Japan, *Jikken Kagaku Koza* (Experimental Chemistry Course), 4th edition, Vol. 19 (1992), Maruzen]. The polytosyl can be obtained by dissolving or suspending the polyol in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide, toluene, tetrahydrofuran, acetonitrile or pyridine, and adding thereto between 1 and 3 molar equivalents (based on the hydroxyl group) of a tosyl halide in the presence of 1～30 mol (based on the hydroxyl group) of an appropriate base (e.g. sodium hydride, zinc oxide, sodium hydroxide, triethylamine or potassium naphthalene), followed by reaction at between -20 and 150°C for between 1 hour and several days.

Then, R^2 is introduced into the obtained mixture containing a branched polyalkylene glycol having two chains or a compound purified therefrom. As R^2, a functional group remaining in a polyol, a polyhalide or a polytosyl can be utilized without modification after polyalkylene glycol A, or, a halide or tosylate thereof is bound to the polyol, polyhalide or polytosyl. Alternatively, a functional group bound to a polyol is protected in advance, and after polyalkylene glycol A or a halide or tosylate thereof is bound, a group obtained by removing the protecting group of the functional group may be utilized as R^2.

In this case, after at least one hydroxyl group or other functional group in the above polyol, polyhalide or polytosyl is protected with an appropriate protective group, polyalkylene glycol A or a halide or tosylate thereof is introduced to the other hydroxyl groups, halogen or tosyl group moiety by the same method as above to synthesize a compound with two polyalkylene glycol chains bound, and then the functional group from which the protective group is removed is utilized as such, or at least one of the functional groups is converted to R^2 according to the method described below. The functional groups present in the polyol, polyhalide or polytosyl before or after binding polyalkylene glycol A or a halide or tosylate thereof include carboxy, amino, halogen, cyano, formyl, carbonyl and the like, in addition to a hydroxyl group. As for the protective groups for functional groups, suitable protective groups for a hydroxyl group include benzyl, tert-butyl, acetyl, benzyloxycarbonyl, tert-butyloxycarbonyl, dimethyl-tert-butylsilyl, diphenyl-tert-butylsilyl, trimethylsilyl, triphenylsilyl, tosyl and tetrahydropyranyl; those for amino include methyl, ethyl, 9-fluorenylmethyloxycarbonyl, benzyloxycarbonyl, nitrobenzyloxycarbonyl, N-phthalimide, acetyl and tert-butyloxycarbonyl; those for carboxy include benzyl, methyl, ethyl, tert-butyl, 9-fluorenylmethyl, methoxyethoxymethyl, 2,2,2-trichloroethyl, 2-(trimethylsilyl)ethyl, cinnamoyl, aryl and nitrophenyl; and those for formyl include dimethyl acetal, diethyl acetal, dibenzyl acetal and 1,3-dioxanyl [Protective Groups In Organic Synthesis, 2nd edition, John Wiley & Sons, Inc. (1991)].

Examples of the polyols, polyhalides and polytosyls having the same aforementioned functional group that can be utilized as R^2, as such or through introduction and removal of a protective group, and being useful as a raw material for constructing the structure of the L moiety include shikimic acid, quinic acid, and Kemp's triacid.

Of the Compounds (I), those compounds which include L and are obtained by adding a new substitution group R^2 can be easily manufactured by the following process for instance.

Process 1-1

Compounds expressed by formula (Iaa) where R^2 of the Compounds (Ia) is a carboxy group

$$(R^1\text{-}M_n\text{-}X^{1a})_2L(X^2\text{-}X^3\text{-}COOH)_q \qquad (Iaa)$$

(wherein X^{1a} is a bond, O, alkylene, $O(CH2)_{ta}$, or $(CH2)_{tb}O$, and R^1, L, M, n, q, X^2 and X^3 are as shown above),
compounds expressed by formula (Iab) where R^2 is carbamoyl

$$(R^1\text{-}M_n\text{-}X^{1a})_2L(X^2\text{-}X^3\text{-}CONH_2)_q \qquad (Iab)$$

(wherein R^1, L, M, n, q, X^{1a}, X^2 and X^3 are the same as shown above), or
compounds expressed by formula (Iac) where R^2 is cyano

$$(R^1\text{-}M_n\text{-}X^{1a})_2L(X^2\text{-}X^3\text{-}CN)_q \qquad (Iac)$$

(wherein R^1, L, M, n, q, X^{1a}, X^2 and X^3 are the same as shown above),
can be synthesized as shown below.

Compounds (Iaa), Compounds (Iab), and Compounds (Iac) can be obtained using a cyclical polyol, for instance by reacting 1 through 30 molar equivalents of acrylic acid, acrylamide, or acrylonitrile or the like with a mixture of reaction products or pure compound containing Compounds (Iaj) which are Compounds (Ia) obtained in accordance with process 1 having a hydroxyl group as R^2, in an appropriate solvent such as water, methylene chloride, toluene, or tetrahydrofuran or the like in the presence of a catalyst or between 1 and 20% base at a temperature of between -20 and 150°C for between one hour and several days. Potassium hydroxide, sodium hydroxide, or sodium hydride may be used as the base.

Furthermore, Compounds (Iaa)can be obtained by hydrolysis after reacting between 1 and 50 molar equivalents of α-halogenated acetate ester with a mixture of reaction products or pure compound containing Compounds (Iaj), which is obtained in accordance with process 1, dissolved or suspended in a nonaqueous condition in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide, toluene, or tetrahydrofuran or the like in the presence of a suitable base such as between 1 and 50 moles of sodium hydride, zinc oxide, sodium hydroxide, or triethylamine at a temperature between -20 and 150°C for between 1 hour and several days.

Furthermore, Compounds (Iaa) can be obtained by dissolving or suspending Compounds (Iaj) obtained in accordance with process 1 in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide, toluene, or tetrahydrofuran or the like, activating and reacting with between 1 and 50 moles of an activating agent such as succinimidyl carbonate, p-nitrophenyl chloroformate, carbonyldiimidazole, or the like, at a temperature between -20 and 100°C for between 1 hour and 10 days, and then reacting with an amino acid such as γ-amino acetate, glysine, β-alanine or derivative thereof.

Furthermore, Compounds (Iaa)can be obtained by reacting Compounds (Iaj) obtained in accordance with process 1 with an acid anhydride such as succinic anhydride or glutaric anhydride, in the presence of a similar base.

Furthermore, Compound (Iaa) can be obtained for instance from a cyclical polyhalide according to process 1, by producing Compounds (Iai) which are Compounds (Ia) where R^2 is a lower level alkyl halide, dissolving or suspending hydroxycarbonate ester, malonic ester, γ-amino acetate ester, β- Alanine ester, or glycine ester or the like in an appropriate solvent such as N,N-dimethyl formamide, dimethyl sulfoxide, toluene, tetrahydrofuran, adding Compounds (Iai) in the presence of between 1 and 50 moles of an appropriate base such as sodium hydride, zinc oxide, sodium hydroxide, or triethylamine, reacting at a temperature of between -20 and 150°C for between 1 hour and several days, and then hydrolyzing.

Furthermore, Compounds (Iaa) can also be obtained, for example, by substituting hydroxyl groups or halogens in one or more locations of said cyclic polyol or cyclic polyhalide with a residue containing carboxylic acid or a carboxylic acid protector, and then substituting the remaining 2 hydroxyl groups or halogens of the cyclic polyol or cyclic polyhalide with polyalkylene glycol group A, or a halogenated compound or tosylated compound thereof using the method shown in process 1.

In this case, introduction of the residue containing carboxylic acid or carboxylic acid protector can be performed in similar manner to the above. In the case of a carboxylic acid protector, polyalkylene glycol group A, or a halogenated compound or tosylated compound thereof is introduced to the cyclic polyol or cyclic polyhalide, and then the protection is removed to generate free carboxylic acid.

Compounds converted to carboxylic acid can be purified and isolated to any arbitrary level of purity by a previously known method such as anion exchange chromatography, hydrophobic chromatography, reverse phase chromatography, two-phase partition, and recrystallization or the like.

Process 1-2

Of Compounds (Ia), those compounds expressed by Equation (Iad) where R^2 is an amino

$$(R^1\text{-}M_n\text{-}X^{1a})_2L(X^2\text{-}X^3\text{-}NH_2)_q \qquad (Iad)$$

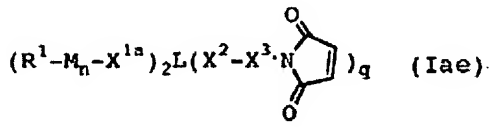
(wherein R^1, L, M, n, q, X^{1a}, X^2 and X^3 are the same as shown above) can be obtained using an appropriate reducing agent on Compounds (Iac) obtained by process 1-1 for instance. The reducing agent may be lithium aluminum hydride, sodium borohydride, sodium cyanoborohydride, or hydrogen or the like.

Furthermore, Compounds (Iad) can be obtained by reacting between 5 equivalents amounts and an excess of a diamine such as ethylene diamine or propylene diamine, with a compound where Compound (Iai) or a halogen component of Compound (Iai) obtained by process 1 for instance is replaced by a tosyl group, in the presence of an appropriate base.

Furthermore, Compounds (Iad) can be obtained through a process similar to that shown in process 1-1, by dissolving or suspending Compound (Iaj) in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide, toluene, or tetrahydrofuran or the like, activating and reacting with 1 to 50 moles of an activating agent such as succinimidyl carbonate, p-nitrophenyl chloroformate, or carbonyldiimidazol or the like in the presence of between 1 and 50 moles of a suitable base such as sodium hydride, zinc oxide, sodium hydroxide, triethylamine or the like at between -20 and 100°C for between 1 hour and 10 days, and then reacting with between 1 equivalent amount and an excess amount of a diamine such as ethylene diamine or propylene diamine in the presence of a suitable base.

Furthermore, Compounds (Iad) can be obtained in conformance with the method shown in process 1 for instance, by introducing an amino or amino protector in one or more locations of a compound such as a cyclic polyol used to form L beforehand, and then substituting the remaining hydroxyl group or halogen component of this compound in 2 locations with polyalkylene glycol group A, or a halogenated compound or tosylated compound thereof.

Of Compounds (Ia), those compounds expressed by Equation (Iae) where R^2 is a maleimide



(Iae)

(wherein R^1, L, M, n, q, X^{1a}, X^2 and X^3 are the same as shown above) can be obtained in accordance with the methods of Oskar Kellar et al. [Helvetica Chim. Acta, Volume 58, page 531 (1975)] or the methods of Timothy P. Kogan et al. [Synthetic Communication, Volume 22, page 2417 (1992)], by reacting the Compounds (Iad) with N-alkoxycarbonyl maleimide in a saturated sodium bicarbonate solution. N-ethoxycarbonyl maleimide or N-methoxycarbonyl maleimide may be used as the N-alkoxycarbonyl maleimide.

Furthermore, Compounds (Iae) can be obtained in conformance with the method shown in process 1 for instance, by introducing maleimide in one or more locations of a compound such as a cyclic polyol or the like used to form L beforehand, and then substituting the remaining hydroxyl group or halogen component of this compound with polyalkylene glycol group A or a halogenated compound or tosylated compound thereof in two locations.

Compounds (Iad) and Compounds (Iac) and synthetic intermediates thereof can be isolated and purified to any arbitrary level of purity by methods similar to those shown above.

Process 1-3

Of the Compounds (Ia), those compounds expressed by Equation (Iaf) where R^2 is formyl

$$(R^1\text{-}M_n\text{-}X^{1a})_2L(X^2\text{-}X^3\text{-}C(=O)H)_q \qquad (Iaf)$$

(wherein R^1, L, M, n, q, X^{1a}, X^2 and X^3 are the same as shown above) can be obtained by oxidation with an appropriate oxidizing agent on Compounds (Iag) which are Compounds (Ia) obtained using process 1 for instance with hydroxymethyl as R^2. Pyridinium chlorochromate, chromic acid, silver ion, and dimethyl sulfoxide or the like may be suggested as the oxidizing agent. Furthermore, Compounds (Iaa) can be obtained by reducing with an appropriate reducing agent in a manner similar to that shown above.

Furthermore, formyl can be introduced by bonding aminoethyl acetal, hydroxyethyl acetal, halogenated ethyl acetal, or halogenated methyl acetal or the like with Compounds (Iaj), Compounds (Iai), or compounds where the halogen components of Compounds (Iai) have been replaced with tosyl groups, obtained by process 1, and then removing the acetal.

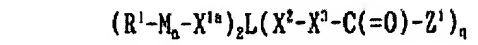
Similarly, formyl can be introduced using Compounds (Iaj) obtained by process 1, by activating the hydroxyl groups in accordance with the method shown in process 1-1, bonding aminoethyl acetal, hydroxyethyl acetal, or the like, and then removing the acetal.

Furthermore, Compounds (Iaf) may also be obtained by a method where aldehyde or aldehyde protector is introduced in one or more locations of a compound such as cyclic polyol or the like used for forming L beforehand, in conformance with the methods shown in process 1 for instance, and then substituting the remaining hydroxyl group or halogen component of that compound in 2 locations with polyalkylene glycol A or halogenated compound or tosylated compound thereof.

Compounds (Iaf) and synthetic intermediates thereof can be isolated and purified to any arbitrary level of purity by methods similar to those shown above.

Process 1-4

Of the Compounds (Ia), those compounds expressed by Equation (Iah) where R^2 is a carbonyl halide

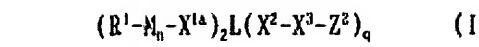


$$(R^1\text{-}M_n\text{-}X^{1a})_2L(X^2\text{-}X^3\text{-}C(=O)\text{-}Z^1)_q \qquad (Iah)$$

(wherein Z^1 is a halogen, and R^1, L, M, n, q, X^{1a}, X^2 and X^3 are the same as shown above) can be obtained by heating Compounds (Iaa) where R^2 is for instance a carboxy, to between 0 and 150°C for between 1 and 24 hours in an appropriate solvent mixture of thionyl halide, or thionyl halide and toluene, or dimethyl formamide, in the presence of an appropriate catalyst such as pyridine or triethylamine or the like.
The halogen in the carbonyl halide is defined the same as aforementioned halogen.

Process 1-5

Of the Compounds (Ia), those compounds expressed by Equation (Iai) where R^2 is a lower alkyl halide



$$(R^1\text{-}M_n\text{-}X^{1a})_2L(X^2\text{-}X^3\text{-}Z^2)_q \qquad (Iai)$$

(wherein Z^2 is a lower alkyl halide, and R^1, L, M, n, q, X^{1a}, X^2 and X^3 are the same as shown above) can be obtained by heating Compounds (Iaj) whereR^2 is for instance a hydroxyl group to between 0 and 150°C for between 1 and 24 hours in an appropriate solvent mixture of thionyl halide, or thionyl halide and toluene, or dimethyl formamide, in the presence of an appropriate catalyst such as pyridine or triethylamine or the like. The halogen and the lower alkyl component in the lower alkyl halide are both defined the same as previously mentioned.

Furthermore, Compounds (Iai) may also be obtained by reacting between 5 equivalent amounts and an excess of a di-halogenated alkyl such as dibromoethane or dibromopropane with Compounds (Iaj) or Compounds (Iad) where R^2 is an amino obtained by Process 1 for instance, in the presence of an appropriate base.

Furthermore, Compounds (Iai) can also be obtained in accordance with the method shown in Process 1 for instance, where a lower alkyl halide is introduced to a compound such as a cyclic polyol or the like used to form L beforehand, and then substituting the remaining hydroxyl group or halogen component of this compound in 2 locations with polyalkylene glycol A or halogenated compound or tosylated compound thereof.

Compounds (Iai) or synthetic intermediates thereof can be isolated and purified to any arbitrary purity by methods similar to those shown above.

Process 1-6

Of the Compounds (Ia), those compounds expressed by Equation (Iak) where R^2 is an isocyanate

$$(R^1\text{-}M_n\text{-}X^{1a})_2L(X^2\text{-}X^3\text{-}N{=}C{=}O)_q \qquad (Iak)$$

(wherein R^1, L, M, n, q, X^{1a}, X^2 and X^3 are the same as shown above) can be obtained for instance by dissolving Compounds (Iad) in an appropriate solvent such as toluene, tetrahydrofuran, or methylene chloride or the like and then reacting with phosgene or oxalyl chloride at between 0 and 150°C for between 1 and 24 hours, or by reacting with N,N'-carbonyl diimidazole and then allowing to decompose at room temperature.

Of the Compounds (Ia), Compounds (Iap) where R^2 is an isothiocyanate can be produced by following the same method as described above, except that thiophosgene is used instead of phosgene.

Process 1-7

Of the Compounds (Ia), those compounds expressed by Equation (Ial) where R^2 is an succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl or phthalimidoxycarbonyl

$(R^1\text{-}M_n\text{-}X^{1a})_2L(X^2\text{-}X^3\text{-}R^{2a})_q$ (Ial)

(wherein R^2 is succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl or phthalimidoxycarbonyl, and R^1, L, M, n, q, X^{1a}, X^2 and X^3 are the same as shown above) can be produced using normal ester synthesis methods. For instance, the target substance can be obtained by reacting between 1 and 10 moles of N-hydroxysuccinimide, substituted or unsubstituted hydroxyallyl, N-hydroxybenzotriazole or N-hydroxyphthalimide with 1 mole of Compound (Iaa) in the presence of between 1 and 10 moles of a condensing agent such as N,N'-dicyclohexylcarbodiimide or the like in an appropriate solvent such as dimethyl formamide, methylene chloride, or dimethyl sulfoxide or the like at a temperature between -20 and 100°C for between 1 and 24 hours. In further detail, according to A. Fradet et al. [Polymer Bulletin, Vol. 4, page 205 (1981)] or K. Geckeler et al. [Polymer Bulletin, Vol. 1, page 691 (1979)], these compounds can be obtained by a method to introduce carboxyl groups to the polyalkylene glycol terminal or a method to produce the N-hydroxysuccinimde ester of carboxymethyl polyalkylene glycol.

Herein, substituted or unsubstituted allyloxycarbonyl is defined as shown above. Allyl is also defined as shown above, and the substituted allyl substitution group is defined in the same manner as the substitution groups in substituted allyloxycarbonyl, substituted allyloxycarbonyloxy, substituted allyldisulfide, and substituted aroyl oxycarbonyl.

Process 1-8

Of the Compounds (Ia), those compounds expressed by Equation (Iam) where R^2 is a vinylsulfonyl

$(R^1\text{-}M_n\text{-}X^{1a})_2L(X^2\text{-}X^3\text{-}SO_2\text{-}CH{=}CH_2)_q$ (Iam)

(wherein R^1, L, M, n, q, X^{1a}, X^2 and X^3 are the same as shown above) can be produced from Compounds (Iaj) for instance using the methods of Margherita Morpurgo et al. [Bioconjugate Chemistry, Vol. 7, page 363 (1996)].

Process 1-9

Of the Compounds (Ia), those compounds expressed by Equation (Ian) where R^2 is a substituted or unsubstituted lower alkoxycarbonyloxy or a substituted or unsubstituted allyloxycarbonyloxy

$$(R^1\text{-}M_n\text{-}X^{1a})_gL(X^2\text{-}X^3\text{-}R^{2b})_q \qquad \text{(Ian)}$$

(wherein R^{2b} is a substituted or unsubstituted lower alkoxycarbonyloxy or a substituted or unsubstituted allyloxycarbonyloxy, and R^1, L, M, n, q, X^{1a}, X^2 and X^3 are the same as shown above) can be obtained for instance by reacting Compounds (Iaj) where R^2 is a hydroxyl group with an excess of p-nitrophenylchloroformate or ethylchloroformate or the like in the presence of a base such as dimethylaminopyridine or triethylamine or the like, in accordance with the methods of Talia Miron and Meir Wilchek [Bioconjugate Chemistry, Volume 4, page 568 (1993)].

Furthermore, Compounds (Ian) can also be obtained using a method as shown in Process 1 for instance, where substituted or unsubstituted alkoxycarbonyloxy or substituted or unsubstituted allyloxycarbonyloxy is introduced to one or more locations on a compound such as cyclic polyol or the like which was used to form L beforehand, and then replacing the remaining hydroxyl groups or halogen component of this compound in two locations with polyalkylene glycol A or halogenated compound or tosylated compound thereof.

Compounds (Ian) and synthetic intermediates thereof can be isolated and purified to any arbitrary purity by methods similar to those shown above.

Here, both substituted or unsubstituted alkoxycarbonyloxy or substituted or unsubstituted allyloxycarbonyloxy are defined as described above.

Process 2: Compounds where X^1 is S

Of Compounds (I), Compounds (Ib) where X^1 is S can be obtained similar to Process 1 by reacting a compound where a cyclic polyol is converted to a cyclic polyhalide [The Chemical Society of Japan, *Jikken Kagaku Koza* (Experimental Chemistry Course) Edition 4, Volume 19 (1992), Maruzen] or a commercial cyclic polyhalide with a thiol derivative of polyalkylene glycol A, in an appropriate solvent in the presence of an appropriate base.

Furthermore, in the reverse of the above process, Compounds (Ib) can be obtained by reacting the halogenated compound or the tosylated compound of polyalkylene glycol A with a cyclic polyol.

The thiol derivative of polyalkylene glycol A may be a commercial product or may be produced by a method compiled by Samuel Zalipsky et al. [Bioconjugate Chemistry, Volume 6, page 150 (1995)].

The reaction conditions and purification conditions for each process shall conform to Process 1.

Process 2-1

Of Compounds (Ib), those compounds where R^2 is a carboxy, carbamoyl, cyano, amino, maleimide, formyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidoxycarbonyl, vinylsulfonyl, substituted or unsubstituted lower alkoxycarbonyloxy, or substituted or unsubstituted allyloxycarbonyloxy, can be obtained by producing a compound where X^1 is -S-in accordance with Process 2, followed by the method described in Process 1-1 through Process 1-9.

Process 3: Compounds where X^1 is NR^3

Of Compounds (I), Compounds (Ic) where X^1 is NR^3 (wherein R^3 is as defined above) can be obtained for instance as described in Process 1, by reacting a compound where a cyclic polyol has been converted to a cyclic polyamine or a commercial cyclic polyamine with a halogenated compound or tosylated compound of polyalkylene glycol A, in an appropriate solvent in the presence of an appropriate base.

Compounds (Ic) can be obtained by reacting an amino derivative of polyalkylene glycol A with a cyclic polyhalide.

Furthermore, Compounds (Ic) can also be obtained by dissolving or suspending 1 equivalent amount of cyclic polyaldehyde with between 1 and 10 equivalents amount of an amino derivative of polyalkylene glycol A in an appropriate solvent such as methanol, ethanol, dimethyl formamide, acetonitrile, dimethyl sulfoxide, water, or buffer solution or the like, and then reacting in the presence of between 1 and 100 equivalent amounts of a reducing agent such as sodium cyanoborohydride or sodium borohydride at a temperature of between -20 and 100°C.

Furthermore, Compound (Ic) can also be produced with using cyclic polyamine and an aldehyde derivative of polyalkylene glycol A.

The cyclic polyaldehyde may be a commercial compound without modification, or an oxidized cyclic polyol or a reduced cyclic polycarboxylic acid. Furthermore, the aldehyde derivative of polyalkylene glycol A may be a commercial compound, or an oxidation of the alcohol at the terminal of polyalkylene glycol A.

The reaction conditions and purification conditions for each process shall conform to Process 1.

Process 3-1

Of Compounds (Ic), those compounds where R^2 is a carboxy, carbamoyl, cyano, amino, maleimide, formyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidoxycarbonyl, vinylsulfonyl, substituted or unsubstituted lower alkoxycarbonyloxy, or substituted or unsubstituted allyloxycarbonyloxy, can be produced by synthesizing Compounds (Ic) in accordance with Process 3, followed by the method described in Process 1-1 through Process 1-9.

Process 4: Compounds where X^1 is R^4-NH-C(=O)-R^5 or R^6-C(=O)-NH-R^7

Of Compound (I), those compounds (Iad) where X^1 is R^4-NH-C(=O)-R^5 (wherein R^4 and R^5 are as defined above) can be obtained in accordance with a peptide synthesis method [Izumiya et al., *Pepuchido Gousei no Kisou to Jikken* (Peptide Synthesis Fundamentals and Experiments) (1985), Maruzen] by dissolving or suspending a cyclic polycarboxylic acid compound selected from Kemp's triacid, cyclohexane tricarboxylic acid, or the like, in an appropriate solvent such as N,N-dimethylformamide or dimethyl sulfoxide or the like, then adding between 1 and 30 equivalent amounts of an alcohol such as N-hydroxysuccinimide, N-hydroxyphthalimide, N-hydroxybenzotriazole, or p-nitrophenol or the like, and between 1 and 30 equivalent amounts of a condensing agent like N,N'-dicyclohexylcarbodiimide, or benzotriazole-1-yloxytripyridinophosphonium hexafluorophosphate or the like, then adding between 1 and 3 equivalent amounts of an amino derivative of polyalkylene glycol A, and reacting. The reaction is performed under anhydrous conditions at between -20°C and 100°C while stirring for between 1 hour and 10 days.

Furthermore, a reaction solution containing high purity double chain branched polyalkylene glycol derivative where R^2 is a carboxy can be obtained by protecting one or more carboxyl groups on a cyclic polycarboxylic acid molecule using an appropriate protector group such as methyl, ethyl, benzyl, or tert-butyl, and then introducing an amino derivative of polyalkylene glycol A to the remaining 2 carboxy groups by the aforementioned method, and then removing the carboxy protector using a usual protector removal method. In this case, introduction of the carboxylic acid protector group and removal of the protector group can be performed using a method which is used for normal peptide synthesis [Izumiya et al., *Pepuchido Gousei no Kisou to Jikken* (Peptide Synthesis Fundamentals and Experiments) (1985), Maruzen]. The arrangement of the carboxy groups on the cyclic polycarboxylic acid may be any arrangement including a three-dimensional arrangement and any average molecular weight of the amino derivative of polyalkylene glycol A may be used so long as the molecular weight distribution is consistent (preferably Mw/Mn is 1.1 or less).

Furthermore, of Compound (I), those compounds (Idb) where X^1 is R^6-C(=O)-NH-R^7 (wherein R^6 and R^7 are as defined above) can be obtained by the reverse of the above process, by reacting a cyclic polyamine with an activated ester of a carboxylic acid derivative of polyalkylene glycol A or an acid halide derivative of polyalkylene glycol A.

The acid halide derivative of polyalkylene glycol A of can be obtained by heating a carboxylic acid derivative of polyalkylene glycol A in an appropriate solvent mixture such as thionyl halide, thionyl halide and toluene, or dimethyl formamide, in the presence of an appropriate catalyst such as pyridine, triethylamine or the like at a temperature of between 0 and 150°C for between 1 and 24 hours.

The reaction conditions and purification conditions for each process shall conform to Process 1.

Process 4-1

Of Compounds (Ida) and Compounds (Idb), those compounds where R^2 is a carboxy, carbamoyl, cyano, amino, maleimide, formyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidoxycarbonyl, vinylsulfonyl, substituted or unsubstituted lower alkoxycarbonyloxy, or substituted or unsubstituted allyloxycarbonyloxy, can be produced by synthesizing Compounds (Ida) or Compounds (Idb) in accordance with Process 4, followed by the method described in Process 1-1 through Process 1-9.

Process 5: Compounds where X^1 is R^8-C(=O)-O or O-C(=O)-R^9

Of Compound (I), those compounds (Ie) where X^1 is R^8-C(=O)-O (where R^8 is as defined above) or O-C(=O)-R^9 (where R^9 is as defined above) can be obtained by dehydration condensation using a combination of polyalkylene glycol A and cyclic polycarboxylic acid, or the carboxylic acid derivative of polyalkylene glycol A and a cyclic polyol. Dehydration condensation methods include those methods normally used for ester synthesis such as dehydration methods in the presence of an acid or base catalyst, or a method of condensing and alcohol and carboxylic acid corresponding to a condensing agent such as N,N'-dicyclohexylcarbodiimide or the like in an appropriate solvent such as dimethylformamide, dimethyl sulfoxide, acetonitrile, pyridine and methylene chloride or the like.

Furthermore, the target material can also be synthesized by using the aforementioned process to react an acidic halogen compound with a corresponding alcohol.

The reaction conditions and purification conditions for each process shall conform to the methods shown in the previous processes.

Process 5-1

Of Compounds (Ie), those compounds where R^2 is a carboxy, carbamoyl, cyano, amino, maleimide, formyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidoxycarbonyl, vinylsulfonyl, substituted or unsubstituted lower alkoxycarbonyloxy, or substituted or unsubstituted allyloxycarbonyloxy, can be produced by synthesizing Compounds (Ie) in accordance with Process 5, followed by the method described in Process 1-1 through Process 1-9.

Process 6: Compounds where X^1 is R^{6a}-O-C(=O)-NH or R^4-NH-C(=O)-O

Of Compounds (I), those compounds (Ifa) where X^1 is R^{6a}-O-C(=O)-NH (where R^{6a} is as defined above) can be produced as shown below for instance.

Raw reaction products including Compounds (Ifa) can be obtained by reacting between 1 and 3 moles of excess carbonate derivative of polyalkylene glycol A with either a commercial cyclic polyamine or a cyclic polyamine produced from cyclic polyol by combining the aforementioned processes. Incidentally, the carbonate derivative of polyalkylene glycol A can be produced in accordance with the methods of Talia Miron et al. (Bioconjugate Chemistry, Volume 4, page 568 (1993)]. Furthermore, N-hydroxysuccimidyl carbonate, p-nitrophenyl carbonate, or imidazolylcarbonyloxy derivative may be used as the carbonate derivative of polyalkylene glycol A.

Of Compounds (I), those compounds (Ifb) where X^1 is R^4-NH-C(=O)-O (where R^4 is as defined above) can be produced as shown below for instance.

Compounds (Ifb) can be obtained by reacting a carbonate derivative of a cyclic polyol with an amino derivative of polyalkylene glycol A, similar to the manner described above.

In accordance with other processes, Compounds (Ifa) or Compounds (Ifb) can selectively be produced by using a combination of functional group protector and protection removal.

The reaction conditions and purification conditions for each process conform to the aforementioned processes.

Process 6-1

Of Compounds (If), those compounds where R^2 is carboxy, carbamoyl, cyano, amino, maleimide, formyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidoxycarbonyl, vinylsulfonyl, substituted or unsubstituted lower alkoxycarbonyloxy, or substituted or unsubstituted allyloxycarbonyloxy, can be produced by synthesizing Compounds (If) in accordance with Process 6, followed by a combination of the methods described in Process 1-1 through Process 1-9.

A double chain compound can be obtained by first obtaining a single chain compound by bonding R^1-M_n-X^1 to L, and then bonding the same or different R^1-M_n-X^1 by the same reaction. For instance, a single chain compound can be obtained by bonding a polyalkylene glycol to a functional group in one location on L using any reaction from Processes 1 through 6. Bonding of the single chain compound produced can be adjusted by changing the ratio of raw material which builds the structure of the L components and the polyalkylene glycol used in the reaction, and a single chain compound may also be used as the main component. The single chain compound obtained may be used in the following step at the original purity, purified to any purity level in accordance with the methods shown in Process 1, or manufactured to a high purity level and used in the next step.

The single chain compound obtained in this manner and the same or a different polyalkylene glycol can be bonded in conformance with any of the methods shown in Processes 1 through 6 to produce a double chain compound.

Incidentally, the second polyalkylene glycol can be attached by the same reaction that was used to make the single chain compound, but using a different reaction to make a formulation with a different bond pattern is also acceptable. For instance, if a compound having a plurality of functional groups such as hydroxyl groups, amino groups or carboxy groups is used as a raw material for building an L component structure, a single chain compound where X^1 is O can be obtained first using the methods shown in Process 1, and then the second polyalkylene glycol can be reacted by the methods shown in Process 4 in order for X^1 to become R^4-NH-C(=O)-O-R^5. As shown above, a double chain compound can be obtained where two polyalkylene glycols are bonded to L by identical or different bond patterns by using a combination of Processes 1 through 6. Furthermore, the average molecular weight of the first and second polyalkylene glycols that were used may be different, and the target compound can easily be achieved by using polyalkylene glycols of different average molecular weights in the reaction where the polyalkylene glycols are bonded to L.

Furthermore, in the reaction where polyalkylene glycol is added to L, it is also possible to leave one or more functional groups in L (for instance, hydroxyl groups in one or more locations for the case of Process 1), protect the other functional groups with an appropriate protector group, and then react to bond with polyalkylene glycol before removing the protector group.

The branched polyalkylene glycol of the present invention can be obtained by the above process even if compounds other than those specifically shown in the aforementioned processes are used.

Incidentally, as described above, the polyalkylene glycol used as a raw material in each step of Processes 1 through 6 may be a commercial product, but it is also possible to easily manufacture it using various methods as compiled by Samuel Zalipsky [Bioconjugate Chemistry, Volume 6, page 150 (1995)].

In defining the various groups used in Equations (Ix), the lower alkyl component on the lower alkyl or lower alkanoyl is straight chain or branched with between 1 and 8 carbons, including for instance, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, pentyl, neopentyl, hexyl, heptyl, or octyl or the like. The alkylene may include for instance methylene, ethylene, n-propylene, isopropylene, n-butylene, isobutylene, sec-butylene, tert-butylene, pentylene, neopentylene, hexylene, heptylene, or octylene or the like.

In Equation (Ix), Mx represents OCH_2CH_2, $OCH_2CH_2CH_2$, $OCH(CH_3)CH_2$, $(OCH_2CH_2)_{rx}$-$(OCH_2CH_2CH_2)_{sx}$ (where rx and sx indicate either the same or different arbitrary positive integer(s)), or $(OCH_2CH_2)_{rax}$-$[OCH(CH_3)CH_2]_{sax}$ (where rax and sax are defined in the same manner as rx and sx, respectively), and if equal to $(OCH_2CH_2)_{rx}$-$(OCH_2CH_2CH_2)_{sx}$ (where rx and sx are defined as shown above, respectively) or $(OCH_2CH_2)_{rax}$-$[OCH(CH_3)CH_2]_{sax}$ (where rax and sax are defined as shown above, respectively), rx and sx as well as rax and sax are preferably between 1 and 100,000, and more preferably between 1 and 1000.

In Equation (Ix), nx represents any positive integer, and is preferably between 10 and 100,000, and more preferably between 100 and 1000.

The polyalkylene glycol component expressed by $(Mx)_{nx}$ of the branched polyalkylene glycol disclosed in Equation (Ix) and made by bonding three or more single chain polyalkylene glycol units, and then bonding either a group which has reactivity towards amino acid site chains in physiological polypeptides, N terminal amino groups, or C terminal carboxyl groups, or a group which can be converted into a group which has said reactivity, preferably has an average molecular weight between approximately 1000 and 1 million, more preferably between 5000 and 100,000. If $(Mx)_{nx}$ is $(OCH_2CH_2)_{nx}$, then the molecular weight distribution expressed by Mw (weight average molecular weight) / Mn (number average molecular weight) of the raw material polyethylene glycol is preferably a simple distribution equal to 1.1 or less, and a commercial product may be used if the average molecular weight is 30,000 or less. For instance, mono-methoxy polypropylene glycol with an average molecular weight of 2000, 5000, 10,000, 12,000, or 20,000 or the like may be used.

In Equation (Ix), qx represents an integer between 1 and 3, and is preferably 1.

In Equation (Ix), mx represents an integer of 3 or higher, and is preferably 3 or 4.

The molecular weight of the branched polyalkylene glycol expressed by Equation (Ix) is preferably in a range between 500 and 1 million.

In Equation (Ix), Lx is a group which can have 4 or more branches, and the substitution groups on Lx may be a hydroxyl group, substituted or unsubstituted lower alkyl, lower alkoxy, amino, carboxy, cyano or formyl or the like.

Here, a lower alkyl or a lower alkyl component of a lower alkoxy is defined the same as the previously mentioned lower alkyl and the substitution groups on the substitution lower alkyl may be a hydroxyl group, amino, lower alkanoyloxy, lower alkanoylamino, lower alkoxy, lower alkoxyalkoxy, lower alkanoyl, lower alkoxycarbonyl, lower alkylcarbamoyl, lower alkylcarbamoyloxy or the like. The lower alkyl component of the lower alkanoyloxy, lower alkanoylamino, lower alkoxy, lower alkoxyalkoxy, lower alkanoyl, lower alkoxycarbonyl, lower alkylcarbamoyl, or lower alkylcarbamoyloxy is defined the same as the aforementioned lower alkyl.

Groups which can have 4 or more branches and are expressed by Lx, and may be in a group which can bond through X^{2x}-X^{3x} to a group which can be converted to a group which has reactivity with an amino side chain in a polypeptide, an N terminal amino group or a C terminal carboxyl group, or to a group which has said reactivity, and may any number of groups may be used as long as the single chain polyalkylene glycol can be branched into 3 molecules or more through X^{1x}. Examples of Lx include groups where four or more hydrogen atoms have been removed from a compound with a molecular weight of 1000 or less, such as polyols or polycarboxylic acid or the like. Specific examples of polyols include lower molecular weight compounds including stereoisomers of glucose, D, L-sorbitol, ribose, erythritol, pentaerythritol, tricine (N-[Tris (hydroxymethyl) methyl] glycine), inositol, choric acid, 3, 4, 5-trihydroxybenzoic acid (Gallic acid), 2, 4, 6-trihydroxybenzoic acid, 3, 4, 5-trihydroxy benzaldehyde, Quinic acid, Shikimic acid, tris (hydroxymethyl) aminomethane or the like, and specific examples of polycarboxylic acid include low molecular weight compounds such as 1, 4, 5, 8-naphthalene tetracarboxylic acid, pyromellitic acid, diethylene triamine pentaacetic acid, 1, 2, 3, 4-butantetra carboxylic acid, citric acid, γ-carboxyglutamine or the like, as well as stereoisomers thereof.

Groups which are preferable for Lx include groups where four or more hydrogen atoms have been removed from tricine, groups where four or more hydrogen atoms have been removed from Shikimic acid, groups where four or more hydrogen atoms have been removed from Quinic acid, groups where four or more hydrogen atoms have been removed from erythritol, groups where four or more hydrogen atoms have been removed from pentaerythritol, and groups where four or more hydrogen atoms have been removed from glucose, or the like.

Construction of the Lx component structure can be achieved using for instance a commercial product compound without modification, or modified with a form appropriate for bonding polyalkylene glycol using a standard organic synthesis method, or used after protecting the functional groups [The Chemical Society of Japan, *Jikken Kagaku Koza* (Experimental Chemistry Course) Edition 4, (1992), Organic Synthesis I ~ V, Maruzen, Protective Groups in Organic Synthesis, Edition 2, John Wiley & Sons, Inc. (1991) or the like].

In addition to the above, cyclohexane can be synthesized using for instance the methods of Kogure et al. [Great Organic Chemistry, Volume 6, page 183 (1958), Asakura Shoten], or G. E. McCasland and E. Clide Horswill [Journal of American Chemical Society, Volume 76, page 2373 (1954)].

In Equation (Ix), the bond between polyalkylene glycol and Lx through X^{1x} can easily be performed using a combination of reactions commonly known for organic synthesis [The Chemical Society of Japan, *Jikken Kagaku Koza* (Experimental Chemistry Course), Edition 4, pages 19 ~ 23 (1992), Organic Synthesis I ~ V, Maruzen].

In Equation (Ix), R^{2x} is a group which has reactivity towards amino acid side chains of polypeptides, N terminal amino groups, or C terminal carboxyl groups, or a group which can be converted into a group having such reactivity.

In other words, groups which have said reactivity include groups which have reactivity towards any of various side chains such as lysine, cystine, arginine, histidine, serine, threonine, tryptophan, asparagic acid, glutamic acid, and glutamine or the like in polypeptides, N terminal amino groups, or C terminal carboxyl groups, and examples of groups having said reactivity include hydroxyl groups, carboxyl, formyl, amino, vinyl sulfonyl, mercapto, cyano, carbamoyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, oxiranyl, lower alkanoyloxy, maleimide, succinimde oxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl, phthalimideoxycarbonyl, imidazolylcarbonyl, substituted or unsubstituted lower alkoxycarbonyloxy, substituted or unsubstituted allyloxycarbonyloxy, tresyl, lower alkanoyloxycarbonyl, substituted or unsubstituted aroyloxycarbonyl, substituted or unsubstituted allyldisulfide, or azido or the like.

In defining the aforementioned groups, the lower alkyl component of the lower alkoxycarbonyloxy, lower alkyl halide, lower alkanoyloxy, and lower alkanoyloxycarbonyl is defined the same as the aforementioned lower alkyl. The allyl component of the allyloxycarbonyl, allyloxycarbonyloxy, and allyldisulfide may be for instance a phenyl, naphthyl, biphenyl, or anthryl, having between 6 and 14 carbons. The aroyl component of the aroyloxycarbonyl may be for instance a benzoyl, naphthoyl, or phthaloyl or the like having between 7 and 13 carbons. The halogen component of the carbonyl halide or the lower alkyl halide may be an atom of fluorine, chlorine, bromine, or iodine.

The substitution groups in the substituted lower alkoxycarbonyloxy may be identical or different groups with the substitution number of between 1 and 3, such as a hydroxyl group, carboxy, or halogen or the like. Here, halogen is as defined above.

The substitution groups in the substituted allyloxycarbonyl, substituted allyloxycarbonyloxy, substituted allyldisulfide, or substituted aroyloxycarbonyl may be identical or different groups with the substitution number of between 1 and 3, such as hydroxyl groups, carboxy, halogen, cyano, or lower alkyl or the like. Here, halogen and lower alkyl are both defined as shown above.

Groups expressed by R^{2x} may be included in the raw materials used to construct the Lx component structure, or may be formed by first protecting the required functional groups in the raw material compound using an appropriate protector [Protective Groups in Organic Synthesis, Edition 2, John Wiley & Sons, Inc. (1991) or the like], creating branches by bonding polyalkylene glycol to Lx through X^{1x}, and then removing the protector and converting as necessary.

Furthermore, after branching the polyalkylene glycol from Lx through X^{1x}, said R^{2x} can also be added to Lx if necessary through X^{2x} or X^{3x} using normal organic synthesis methods.

More specifically, the polyalkylene glycol of the present invention can be produced for instance by the following processes, but the production method is not restricted to these examples.

Process 1x: Production of compounds wherein X^{1x} is a bond, O, alkylene, $O(CH_2)_{tax}$, or $(CH_2)_{tbx}O$

Of Compounds (Ix), those compounds (Iax) where X^{1x} is a bond, O, alkylene, $O(CH_2)_{tax}$ (wherein tax is defined the same as above), or $(CH_2)_{tbx}O$ (wherein tbx is defined the same as above) can be produced for instance by the following methods.

Mixtures containing branched polyalkylene glycol with 3 or more chains can be obtained by dissolving or suspending in an anhydrous condition a polyol with hydroxyl groups in three or more locations in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide, toluene, tetrahydrofuran, acetonitrile, or pyridine or the like in the presence of between 1 and 30 moles of an appropriate base such as sodium hydride, zinc oxide, sodium hydroxide, triethylamine or the like, and then adding 3 or more moles of halogenated or tosylated compounds of polyalkylene glycol or monoalkyl ester or monocarbonyl ester thereof (hereinafter polyalkylene glycol or monoalkyl ester or monocarbonyl ester thereof is referred to in whole as polyalkylene glycol Ax), and then reacting at a temperature of between -20 and 150°C for between 1 hour and 10 days.

The polyol may be selected from commercial compounds such as quinic acid, glucose, sorbitol, ribose, erythritol, penterythritol, tricine, and inositol or the like, or compounds derived from these commercial compounds. Compounds derived from these commercial compounds include polyol or the like derived for instance by reducing a carboxylic acid selected from ethylenediaminetetraacetic acid, 1,2,4,5-benzenetetracarboxylic acid, and γ-carboxyglutamic acid and the like, with an appropriate reducing agents in accordance with normal organic synthesis methods[The Chemical Society of Japan, *Jikken Kagaku Koza* (Experimental Chemistry Course), Edition 4, Volumes 19-21 (1992), Maruzen].

The reducing agent may be lithium aluminum hydride, sodium borohydride, sodium cyanoborohydride, or hydrogen or the like.

Any arrangement of hydroxy groups in the polyol is acceptable, and the reaction may be performed after protecting functional groups which are not necessary in the reaction according to methods shown in the literature [Protective Groups in Organic Synthesis, Edition 2, John Wiley & Sons, Inc. (1991)], or after forming a derivative.

Halogenated or tosylated compounds of polyalkylene glycol Ax can be easily produced using the various methods disclosed in Samuel Zalipsky's compilation [Bioconjugate Chemistry, Volume 6, page 150 (1995)] or the like. The halogenated compound or tosylated compound of the polyalkylene glycol Ax used for bonding may have any average molecular weight so long as the molecular weight distribution is consistent (Mw/Mn is preferably 1.1 or less).

The obtained mixture containing branched polyalkylene glycol with three or more chains may be used in the following steps at the original purity, or branched polyalkylene glycol with 3 chains, 4 chains, 5 chains, or more chains may be isolated and purified to any level of purity using conventionally known methods such as ion exchange chromatography, reverse phase chromatography, hydrophobic chromatography, two-phase separation, or recrystallization or the like. In the previous steps, any number of compounds (Iajx) where R^2 is a hydroxyl group can be obtained based on compounds (Iax).

On the other hand, the target branched polyalkylene glycol with three or more chains can be produced even when using polyalkylene glycol Ax with polyhalides or polytosyls. In this case, three or more equivalent moles of polyalkylene glycol Ax are dissolved or suspended in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide, toluene, or tetrahydrofuran or the like, in the presence of between 1 and 30 moles of an appropriate base such as sodium hydride, zinc oxide, sodium hydroxide, or triethylamine or the like per mole of polyalkylene glycol Ax, and then 1 equivalent mole of a polyhalide or polytosyl are added and reacted at a temperature of between -20 and 150°C for between 1 hour and 10 days, in order to obtain the target substance.

The polyhalide may be a commercial compound or can be obtained by modifying a polyol to a halogenated compound [The Chemical Society of Japan, *Jikken Kagaku Koza* (Experimental Chemistry Course), Edition 4, Volume 19 (1992), Maruzen]. The polytosyl can be obtained by dissolving or suspending a polyol in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide, toluene, tetrahydrofuran, acetonitrile, or pyridine or the like, in the presence of between 1 and 30 moles per hydroxyl group of an appropriate base such as sodium hydride, zinc oxide, sodium hydroxide, triethylamine, or potassium naphthalene, adding between 1 and 3 equivalent moles per hydroxyl group of halogenated tosyl, and then reacting at a temperature of between -20 and 150°C for between 1 hour and several days.

Next, R^{2x} is introduced to a mixture or pure compound containing the branched polyalkylene glycol with three or more chains that was obtained. The R^{2x} may be polyalkylene glycol Ax or halogenated compound or tosylated compound thereof, bonded to a polyol, polyhalides, or polytosyl, and then used without modification to the functional groups remaining on the polyol, polyhalides, or polytosyl, or it is also acceptable that the functional groups bonded to the polyol be protected beforehand, and then the polyol is bonded to the polyalkylene glycol A or halogenated compound or tosylated compound thereof, after which the protector group for the functional group is removed. In this case, one or more hydroxyl groups or other functional group on the polyol, polyhalides, or polytosyl is protected by an appropriate protective group, and then polyalkylene glycol Ax or halogenated or tosylated compound thereof is introduced to the remaining hydroxyl groups, halogen, or tosyl group component using the same methods as described above, in order to synthesize a compound where three or more polyalkylene glycol are bonded, and the compound may be used with the protector groups removed without modifying the functional groups, or at least one of the functional groups may be converted to R^{2x} in accordance with methods described below. The functional groups which exist on the polyol, polyhalides, or polytosyl prior to or after bonding with the polyalkylene glycol Ax or the halogenated or tosylated compound thereof, may be a hydroxyl group, or carboxy, amino, halogen, cyano, formyl, or carbonyl, or the like. Appropriate protectors for the functional groups may be benzyl, tert-butyl, acetyl, benzyloxycarbonyl, tert-butyloxycarbonyl, methyl-tert-butylsilyl, diphenyl-tert-butylsilyl, trimethylsilyl, triphenylsilyl, tosyl, or tetrahydropyranyl or the like for the case of hydroxyl groups; methyl, ethyl, 9-fluorenylmethyloxycarbonyl, benzyloxycarbonyl, nitrobenzyloxycarbonyl, N-phthalimide, acetyl, tert-butyloxycarbonyl or the like, for the case of amino; benzyl, methyl, ethyl, tert-butyl, 9-fluorenylmethyl, methoxyethoxymethyl, 2,2,2-trichloroethaneethyl, 2-(trimethylsilyl) ethyl, cinnamoyl, allyl, nitrophenyl or the like, for the case of carboxy; and dimethyl acetal, diethyl acetal, dibenzyl acetal, 1,3-dioxanyl or the like, for formyl

[Protective Groups in Organic Synthesis, Edition 2, John Wiley & Sons, Inc. (1991)].

Previously existing functional groups may be used without modification, or may be used as R^{2x} after protecting and removing protection, and specific examples of the polyol, polyhalides, or polytosyl which are the raw materials for constructing the Lx component structure include shikimic acid, quinic acid, 3,4,5-trihydroxybenzoic acid, choric acid or halide or tosylated compounds thereof.

Of Compounds (Ix), compounds which are obtained by introducing a new substitution group R^{2x} to compounds containing Lx can easily be produced by the following processes.

Process 1x-1

Of Compounds (Iax), those compounds expressed by Equation (Iaax) where R^{2x} is carboxy

$$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1ax})_{mx}Lx(X^{2x}\text{-}X^{3x}\text{-}COOH)_{qx} \qquad \text{(Iaax)}$$

(wherein X^{1ax} represents a bond, O, alkylene, $O(CH_2)_{tax}$, or $(CH_2)_{tbx}O$, and R^{2x}, Lx, Mx, nx, mx, qx, X^{2x}, and X^{3x} are as defined above)

Those compounds expressed by Equation (Iabx) where R^{2x} is carbamoyl

$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1ax})_{mx}Lx(X^{2x}\text{-}X^{3x}\text{-}CONH_2)_{qx}$ (Iabx)

(wherein, R^{1x}, Lx, Mx, nx, mx, qx, X^{2x}, and X^{3x} are each as defined above), and those compounds expressed by Equation (Iacx) where R^{2x} is cyano

$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1ax})_{mx}Lx(X^{2x}\text{-}X^{3x}\text{-}CN)_{qx}$ (Iacx)

(wherein, R^{1x}, Lx, Mx, nx, mx, qx, X^{2x}, and X^{3x} are each as defined above) can be synthesized for instance as shown below.

Compounds (Iaax), Compounds (Iabx), and Compounds (Iacx) can be obtained by reacting a reaction product mixture or purified compound containing compounds (Iajx) which are compounds (Iax) obtained using a polyol by following Process 1x and which have hydroxyl groups as R^{2x}, with between 1 and 30 moles equivalent of acrylic acid, acrylamide, or acrylonitrile or the like in an appropriate solvent such as water, methylene chloride, toluene, or tetrahydrofuran or the like, in the presence of a catalyst or between 1 and 20% base, at a temperature between -20 and 150°C for between 1 hour and several days. Potassium hydroxide, sodium hydroxide, or sodium hydride or the like may be used as the base. Furthermore, Compounds (Iaax) can be obtained by dissolving or suspending a reaction product mixture or purified compound including compounds (Iajx) obtained for instance by Process 1x, in an anhydrous condition in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide, toluene, or tetrahydrofuran or the like, in the presence of between 1 and 50 moles of an appropriate base such as sodium hydride, zinc oxide, sodium hydroxide, or triethylamine or the like, reacting with between 1 and 50 equivalents of α-halogenated acetate ester at a temperature of between -20 and 150°C for between 1 hour and several days, and then hydrolyzing. Furthermore, Compounds (Iaax) can also be obtained by dissolving or suspending Compounds (Iajx) obtained for instance by process 1x in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide, toluene, or tetrahydrofuran or the like, and then activating and reacting with between 1 and 50 moles of an activating agent such as succinimidyl carbonate, p-nitrophenyl chloroformate, carbonyldiimidazole, or the like, in the presence of between 1 and 50 moles of an appropriate base such as sodium hydride, zinc oxide, sodium hydroxide, or triethylamine or the like, at a temperature of between -20 and 100°C for between 1 hour and 10 days, and then reacting with an amino acid such as γ-aminoacetic acid, glycine, or β-alanine or a derivative thereof.

Furthermore, Compounds (Iaax) can also be produced by reacting compounds (Iajx) obtained by Process 1x with an acidic anhydride such as succinic anhydride or glutaric anhydride in the presence of the aforementioned base.

Furthermore, Compounds (Iaax) can be obtained by producing compound (Iaix) which is a compound (Iax) where R^{2x} is a lower alkyl halide using a polyhalide in accordance with Process 1x, and then dissolving or suspending hydroxycarboxylic acid ester, malonic acid ester, γ-amino acetate ester, β-alanine ester, or glycine ester or the like in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide, toluene, or tetrahydrofuran, adding Compound (Iaix) in the presence of between 1 and 50 moles of an appropriate base such as sodium hydride, zinc oxide, sodium hydroxide, or triethylamine or the like, reacting at a temperature between -20 and 150°C for between 1 hour and several days, and then hydrolyzing.

Furthermore, Compound (Iaax) can also be obtained by first substituting a hydroxyl group or halogen in one or more locations of a polyol or polyhalide with carboxylic acid or a residue group which contains a carboxylic acid protector, and then substituting the hydroxyl groups or halogens in the remaining three or more locations of the polyolefin or polyhalide with polyalkylene glycol Ax or halogenated compound or tosylated compound thereof using the method shown in Process 1. In this case, the introduction of the carboxylic acid protector or the residue group containing a carboxylic acid protector can be performed in a manner similar to as previously described. If the carboxylic acid has been protected, the protection is removed after introducing the polyalkylene glycol Ax or the halogenated compound or tosylated compound thereof to the polyol or polyhalide to produce free carboxylic acid.

Compounds with substituted carboxylic acid can be purified and isolated to any level of purity using a conventionally known method such as anionic exchange chromatography, hydrophobic chromatography, reverse phase chromatography, two-phase separation, or a crystallization or the like.

Process 1x-2

Of Compounds (Iax), those compounds expressed by Equation (Iadx) where R^{2x} is an amino

$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1ax})_{mx}Lx(X^{2x}\text{-}X^{3x}\text{-}NH_2)_{qx}$ (Iadx)

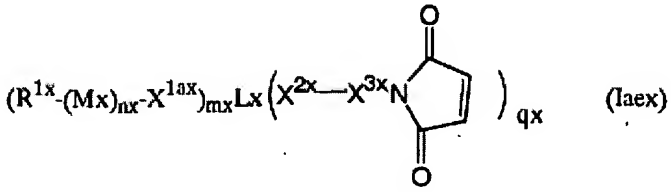
(wherein, R^{1x}, Lx, Mx, nx, mx, qx, X^{1ax}, X^{2x}, and X^{3x} are each as defined above) can be obtained by using an appropriate reducing agent on compounds (Iacx) obtained for instance using Process 1x-1. The reducing agent may be lithium aluminum hydride, sodium borohydride, sodium cyanoborohydride, or hydrogen or the like.

Furthermore, Compounds (Iadx) can also be obtained by reacting between 5 equivalent amounts and an excess amount of a diamine such as ethylene diamine or propylene diamine or the like with the compound (Iaix) obtained by Process 1x-1 for instance or a compound where the halogen component of compound (Iaix) has been substituted with a tosyl group, in the presence of an appropriate base.

Furthermore, similar to the manner shown in Process 1x-1, Compound (Iadx) can be obtained by dissolving or suspending for instance compound (Iajx) in an appropriate solvent such as N,N-dimethylformamide, dimethyl sulfoxide, toluene, or tetrahydrofuran or the like, and then activating by reacting with between 1 and 50 moles of an activating agent such as succinimidyl carbonate, p-nitrophenyl chloroformate, carbonyldiimidazole, or the like at a temperature of between -20 and 100°C for between 1 hour and 10 days, and then reacting with between 1 equivalent amount and an excess amount of a diamine such as ethylene diamine or propylene diamine or the like, in the presence of an appropriate base such as between 1 and 50 moles of sodium hydride, zinc oxide, sodium hydroxide, or triethylamine.

Furthermore, Compound (Iadx) can also be obtained by a method where an amino or an amino protector is introduced in one or more locations to a compound such as a polyol which is used for forming Lx beforehand, in accordance with the method shown in Process 1x for instance, and then substituting the remaining hydroxyl groups or halogen components of this compound in three or more locations with polyalkylene glycol Ax or a halogenated compound or tosylated compound thereof.

Of Compounds (Iax), those compounds expressed by Equation (Iaex) where R^{2x} is maleimide



(Iaex)

(wherein, R^{1x}, Lx, Mx, nx, mx, qx, X^{1ax}, X^{2x}, and X^{3x} are each as defined above) can be obtained for instance by reacting Compounds (Iadx) with N-alkoxycarbonylmaleimide in a saturated sodium bicarbonate aqueous solution, in accordance with the methods of Oskar Keller et al. [Helvetica Chim. Acta, Volume 58, page 531 (1975)] or Timothy P. Kogan et al. [Synthetic Communication, Volume 22, page 2417 (1992)]. N-ethoxycarbonylmaleimide or N-ethoxycarbonylmaleimide may be used as the N-alkoxycarbonylmaleimide.

Furthermore, Compounds (Iaex) can also be obtained by a method where maleimide is introduced at one or more locations on a compound such as polyol which was used for forming Lx beforehand, in accordance with the method shown in Process 1x for instance, and then substituting the remaining hydroxyl group or halogen component of this compound in three or more locations with polyalkylene glycol Ax or a halogenated compound or tosylated compound thereof.

Compounds (Iadx), Compounds (Iaex), and synthetic intermediates thereof can be isolated and purified to any arbitrary level of purity corresponding to the number of branches of the polyalkylene glycol using the same methods as described above.

Process 1x-3

Of Compounds (Iax), those compounds expressed by Equation (Iafx) where R^{2x} is formyl

$$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1ax})_{mx}Lx(X^{2x}\text{-}X^{3x}\text{-}C(=O)H)_{qx} \qquad \text{(Iafx)}$$

(wherein, R^{1x}, Lx, Mx, nx, mx, qx, X^{1ax}, X^{2x}, and X^{3x} are each as defined above) can be obtained by oxidizing Compounds (Iagx), which are Compounds (Iax) obtained for instance by Process 1x and having hydroxymethyl as R^{2x}, with an appropriate oxidizing agent. Pyridium chromate, chromic acid, silver ion, or dimethyl sulfoxide or the like may be suggested as the oxidizing agent. Furthermore, as described above, Compounds (Iaax) can also be obtained by reducing with an appropriate reducing agent.

Furthermore, formyl can be introduced to Compounds (Iajx), Compounds (Iaix), or compounds where the halogen component in Compounds (Iaix) has been substituted by a tosyl group by bonding aminoethyl acetal, hydroxy ethyl acetal, halogenated ethyl acetal, or halogenated methyl acetal or the like, and then removing the acetal.

Similarly, formyl can also be introduced using Compounds (Iajx) obtained by Process 1x by activating the hydroxyl group by the method shown in Process 1x-1, and then bonding aminoethyl acetal or hydroxyethyl acetal or the like and then removing the acetal.

Furthermore, Compounds (Iafx) can also be obtained by a method where an aldehyde or aldehyde protector is introduced in one or more locations to a compound such as a polyol which was used for forming Lx beforehand in accordance with the method shown in Process 1x for instance, and then substituting the remaining hydroxyl groups or halogen components of this compound in three or more locations with polyalkylene glycol Ax or a halogenated compound or tosylated compound thereof.

Compounds (Iafx) and synthetic intermediates thereof can be isolated and purified to any arbitrary level of purity corresponding to the number of branches of the polyalkylene glycol using the same methods as described above.

Process 1x-4

Of Compounds (Iax), those compounds expressed by Equation (Iahx) where R^{2x} is carbonyl halide

$$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1ax})_{mx}Lx(X^{2x}\text{-}X^{3x}\text{-}C(=O)\text{-}Z^{1x})_{qx} \qquad \text{(Iahx)}$$

(wherein, Z^{1x} represents a halogen, and R^{1x}, Lx, Mx, nx, mx, qx, X^{Iax}, X^{2x}, and X^{3x} are each defined as described above) can be obtained for instance by heating Compounds (Iaax) where R^{2x} is carboxy in an appropriate solvent mixture of thionyl halide, thionyl halide and toluene, or dimethyl formamide or the like, in the presence of an appropriate catalyst such as pyridine or triethylamine or the like, at a temperature of between 0 and 150°C for between 1 and 24 hours. The halogen in the carbonyl halide is defined the same as previously described halogens.

Process 1x-5

Of Compounds (Iax), those compounds expressed by Equation (Iaix) where R^{2x} is lower alkyl halide

$$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1ax2})_{mx}Lx(X^{2x}\text{-}X^{3x}\text{-}Z^{2x})_{qx} \qquad \text{(Iaix)}$$

(wherein, Z^{2x} represent a lower alkyl halide, and R^{1x}, Lx, Mx, nx, mx, qx, X^{Iax}, X^{2x}, and X^{3x} are each defined as described above) can be obtained by heating Compound (Iajx), where R^{2x} is for instance a hydroxyl group, in an appropriate solvent mixture such as thionyl halide, thionyl halide and toluene, or dimethyl formamide or the like, in the presence of an appropriate catalyst such as pyridine or triethylamine or the like, at a temperature of between 0 and 150°C for between 1 and 24 hours. The halogen in the lower alkyl halide and the lower alkyl component are defined the same as previously described.

Furthermore, Compounds (Iaix) can also be obtained by reacting Compounds (Iaix) or Compounds (Iadx) where R^{2x} is an amino, which were obtained by Process 1x for instance with between 5 equivalent parts and an excess amount of a dihalogenated alkyl such as dibromoethane or dibromopropane or the like in the presence of an appropriate base as described above.

Furthermore, Compound (Iaix) can also be obtained by a method where a lower alkyl halide is introduced in one or more locations to a compound such as a polyol which was used for forming Lx beforehand in accordance with the method shown in Process 1x for instance, and then substituting the remaining hydroxyl groups or halogen components of this compound in three or more locations with polyalkylene glycol Ax or a halogenated compound or tosylated compound thereof.

Compounds (Iaix) and synthetic intermediates thereof can be isolated and purified to any arbitrary level of purity corresponding to the number of branches of the polyalkylene glycol using the same methods as described above.

Process 1x-6

Of Compounds (Iax), those compounds expressed by Equation (Iakx) where R^{2x} is isocyanate

$$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1ax})_{mx}Lx(X^{2x}\text{-}X^{3x}\text{-}N{=}C{=}O)_{qx} \qquad \text{(Iakx)}$$

(wherein, R^{1x}, Lx, Mx, nx, mx, qx, X^{1ax}, X^{2x}, and X^{3x} are each defined as described above) can be obtained by reacting Compounds (Iadx) for instance with phosgene or oxalyl chloride in an appropriate solvent such as toluene, tetrahydrofuran, or methylene chloride or the like at a temperature of between 0 and 150°C for between 1 and 24 hours, or by reacting with N,N'-carbonyldiimidazole and then causing to decompose at room temperature.

Of Compounds (Iax), those compounds (Iapx) where R^{2x} is isothiocyanate (-N =C =S) can be obtained by the above method except that thiophosgene is used in place of phosgene.

Process 1x-7

Of Compounds (Iax), those compounds expressed by Equation (Ialx) where R^{2x} represents succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl or phthalimidoxycarbonyl

$$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1ax})_{mx}Lx(X^{2x}\text{-}X^{3x}\text{-}R^{2ax})_{qx} \qquad \text{(Ialx)}$$

(wherein, R^{2x} represents succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl or phthalimidoxycarbonyl, and R^{1x}, Lx, Mx, nx, mx, qx, X^{1ax}, X^{2x}, and X^{3x} are each defined as described above) can be produced using normal ester synthesis methods.

For instance, the target substance can be obtained by reacting between 1 and 10 moles of N-hydroxysuccinimide, substituted or unsubstituted allyl hydroxide, N-hydroxybenzotriazole, or N-hydroxyphthalimide with 1 mole of Compound (Iaax) in the presence of between 1 and 10 moles of a condensing agent such as N,N'-dicyclohexylcarbodiimide or the like in an appropriate solvent such as dimethylformamide, methylene chloride, or dimethyl sulfoxide or the like, at a temperature of between -20 and 100°C for between 1 and 24 hours. In further detail, the target substance can be obtained by introducing a carboxyl group to the polyalkylene glycol terminal in accordance with A. Fradet et al. [Polymer Bulletin, Volume 4, page 205 (1981)], or K. Geckeler et al. [Polymer Bulletin, Volume 1, page 691 (1979)], or in conformance with the production method for the N-hydroxysuccinimide ester of carboxymethyl polyalkylene glycol.

Here, substituted or unsubstituted allyloxycarbonyl is defined as described above. The allyl component of the allyl hydroxide is defined the same as the allyl component of the allyloxycarbonyl, and the substitution group of substituted allyl hydroxide is defined the same as the substitution group for substituted allyloxycarbonyl.

Process 1x-8

Of Compounds (Iax), those compounds expressed by Equation (Iamx) where R^{2x} is vinylsulfonyl

$$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1ax})_{mx}Lx(X^{2x}\text{-}X^{3x}\text{-}SO_2\text{-}CH{=}CH_2)_{qx} \qquad \text{(Iamx)}$$

(wherein, R^{1x}, Lx, Mx, nx, mx, qx, X^{1ax}, X^{2x}, and X^{3x} are each defined as described above) can be produced using Compounds (Iajx) for instance by the methods of Margherita Morpurgo et al. [Bioconjugate Chemistry, Volume 7, page 363 (1996)].

Process 1x-9

Of Compounds (Iax), those compounds expressed by Equation (Ianx) where R^{2x} represents substituted or unsubstituted lower alkoxycarbonyloxy or substituted or unsubstituted allyloxycarbonyloxy

$$(R^{1x}\text{-}(Mx)_{nx}\text{-}X^{1ax})_{mx}Lx(X^{2x}\text{-}X^{3x}\text{-}R^{2bx})_{qx} \qquad \text{(Ianx)}$$

(wherein, R^{2x} represents substituted or unsubstituted lower alkoxycarbonyloxy or substituted or unsubstituted allyloxycarbonyloxy, and R^{1x}, Lx, Mx, nx, mx, qx, X^{1ax}, X^{2x}, and X^{3x} are each defined as described above) can be obtained by reacting Compounds (Iajx) where R^{2x} is a hydroxy group with an excess of p-nitrophenylchloroformate or ethylchloroformate or the like in the presence of a base such as dimethylaminopyridine or triethylamine or the like, in accordance with the methods of Talia Miron and Meir Wilchek [Bioconjugate Chemistry, Volume 4, page 568 (1993)].

Furthermore, Compound (Ianx) can also be obtained by a method where a substituted or unsubstituted alkoxycarbonyloxy or substituted or unsubstituted allyloxycarbonyloxy is introduced in one or more locations to a compound such as a polyol which was used for forming Lx beforehand in accordance with the method shown in Process 1x for instance, and then substituting the remaining hydroxyl groups or halogen components of this compound in three or more locations with polyalkylene glycol Ax or a halogenated compound or tosylated compound thereof.

Compounds (Ianx) and synthetic intermediates thereof can be isolated and purified to any arbitrary level of purity corresponding to the number of branches of the polyalkylene glycol using the same methods as described above.

Here, substituted or unsubstituted alkoxycarbonyloxy or substituted or unsubstituted allyloxycarbonyloxy are defined as described above.

Process 2x: Compounds where X^{1x} represents S

Of Compound (Ix), those compounds (Ibx) where X^{1x} represents S can be obtained in a manner similar to that described in Process 1x, for instance, by reacting a compound where a polyol is converted to a poly halide[The Chemical Society of Japan, *Jikken Kagaku Koza* (Experimental Chemistry Course), Edition 4, Volume 19 (1992), Maruzen] or a commercial polyhalide, with a thiol derivative of polyalkylene glycol Ax in an appropriate solvent in the presence of an appropriate base.

Furthermore, Compounds (Ibx) can be obtained by the reverse process by reacting a halogenated compound or tosylated compound of polyalkylene glycol Ax with a polythiol.

The thiol derivative of polyalkylene glycol Ax may be a commercial product, or may be produced by a method summarized by Samuel Zalipsky et al. [Bioconjugate Chemistry, Volume 6, page 150 (1995)].

The reaction conditions and purification conditions for each process shall conform to Process 1x.

Process 2x-1

Of Compounds (Ibx), those compounds where R^{2x} represents carboxy, carbamoyl, cyano, amino, maleimide, formyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidoxycarbonyl, vinylsulfonyl, substituted or unsubstituted lower alkoxycarbonyloxy, or substituted or unsubstituted allyloxycarbonyloxy, can be produced by synthesizing a compound where X^{1x} represents S in accordance with Process 2x, followed by the method described in Process 1x-1 through Process 1x-9.

Process 3x: Compounds where X^{1x} represents NR^{3x}

Of Compounds (Ix), those compounds (Icx) where X^{1x} represents NR^{3x} (wherein R^{3x} is defined as described above) can be obtained by reacting a commercial polyamine or a compound where a polyol has been converted to a polyamine similarly to Process 1x, with the halogenated compound or tosylated compound of polyalkylene glycol Ax in an appropriate solvent in the presence of an appropriate base.

Compounds (Icx) can be obtained by reacting amino derivative of polyalkylene glycol Ax with a poly halide.

Furthermore, Compounds (Icx) can be obtained by dissolving or suspending one equivalent amount of polyaldehyde and an amino derivative of polyalkylene glycol Ax (the amino derivative of the polyalkylene glycol Ax is present in a ratio of between 1 and 30 equivalent amounts per formyl group on the poly aldehyde) in an appropriate solvent such as methanol, ethanol, dimethyl formamide, acetonitrile, dimethyl sulfoxide, water, or buffer solution or the like, and then reacting in the presence of a reducing agent such as sodium cyanoborohydride or sodium borohydride or the like (the reducing agent is present in a ratio of between 1 and 30 equivalent amounts per formyl group on the poly aldehyde) at a temperature of between -20 and 100°C.

Furthermore, Compound (Icx) can be produced using a polyamine and the aldehyde derivative of polyalkylene glycol Ax.

The polyaldehyde may be a commercial compound without modification, or may be an oxidized polyalcohol or may be a reduced polycarboxylic acid. Furthermore, the aldehyde derivative of the polyalkylene glycol Ax may be a commercial compound or may be an oxidized alcohol on the terminus of the polyalkylene glycol Ax.

The reaction conditions and purification conditions for each process shall conform to Process 1x.

Process 3x-1

Of Compounds (Icx), those compounds where R^{2x} is a carboxy, carbamoyl, cyano, amino, maleimide, formyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidoxycarbonyl, vinylsulfonyl, substituted or unsubstituted lower alkoxycarbonyloxy, or substituted or unsubstituted allyloxycarbonyloxy, can be obtained by synthesizing a compound (Icx) in accordance with Process 3x, followed by the method described in Process 1x-1 through Process 1x-9.

Process 4x: Compounds where X^{1x} represents R^{4x}-NH-C(=O)-R^{5x} or R^{6x}-C(=O)-NH-R^{7x}

Of Compounds (Ix), those compounds (Idax) where X^{1x} represents R^{4x}-NH-C(=O)-R^{5x} (wherein R^{4x} and R^{5x} are both defined as described above) can be obtained by dissolving or suspending a polycarboxylic acid compound selected from γ-carboxyglutamic acid, citric acid, and 1,2,3,4-butantetracarboxylic acid and the like in an appropriate solvent such as N,N-dimethylformamide, or dimethyl sulfoxide, in accordance with peptide synthesis methods [Izumiya et al., *Pepuchido Gousei no Kisou to Jikken* (Peptide Synthesis Fundamentals and Experiments) (1985), Maruzen], and then adding between 1 and 30 equivalent amounts of an alcohol such as N-hydroxysuccinimide, N-hydroxyphthalimide, N-hydroxybenzotriazole, or p-nitrophenol or the like for each carboxyl group on the polycarboxylic acid compound, and also adding between 1 and 30 equivalent amounts of a condensing agent such as N,N'-dicyclohexylcarbodiimide, benzotriazole-1-yloxytripyridinophosphonium hexafluorophosphate, or the like for each carboxyl group on the polycarboxylic acid compound, and then adding and reacting between one and 30 equivalent amounts of an amino derivative of polyalkylene glycol Ax for each carboxyl group on the polycarboxylic acid compound. The reaction is performed under anhydrous conditions at a temperature of between -20 and 100°C for between 1 hour and 10 days, while mixing.

Furthermore, a reaction solution containing a high purity level of branched polyalkylene glycol derivative with three or more chains where R^{2x} represents carboxy can be obtained by protecting one or more carboxy on the polycarboxylic acid molecule using an appropriate protective group such as methyl, ethyl, benzyl, or tert-butyl, or the like, introducing an amino derivative of polyalkylene glycol Ax at the remaining carboxy using the aforementioned methods, and then removing the carboxy protective groups using standard protection removing methods. In this case, the introduction of the carboxylic acid protective group and removal of that protective group can be performed using a method used for normal poly peptide synthesis [Izumiya et al., *Pepuchido Gousei no Kisou to Jikken* (Peptide Synthesis Fundamentals and Experiments) (1985), Maruzen]. The carboxy on the polycarboxylic acid may have any arrangement including a three-dimensional arrangement, and any average molecular weight is acceptable so long as the molecular weight distribution of the amino derivative of the polyalkylene glycol Ax is uniform (Mw/Mn is preferably 1.1 or less).

Of Compounds (Ix), those compounds (Idbx) where X^{1x} represents R^{6x}-C(=O)-NH-R^{7x} (wherein R^{6x} and R^{7x} are both defined as described above) can be obtained by the reverse process to the above, by reacting a polyamine with an active ester of a carboxylic acid derivative of polyalkylene glycol Ax or an acid halide derivative of polyalkylene glycol Ax. The acid halide derivative of polyalkylene glycol Ax can be obtained by heating a carboxylic acid derivative of polyalkylene glycol Ax in an appropriate solvent mixture such as thionyl halide, thionyl halide and toluene, or dimethyl formamide or the like, in the presence of an appropriate catalyst such as pyridine or triethylamine or the like, at a temperature of between 0 and the 150°C for between 1 and 24 hours.

The reaction conditions and purification conditions for each process shall conform to the aforementioned methods.

Process 4x-1

Of Compounds (Idax) and Compound (Idbx), those compounds where R^{2x} is carboxy, carbamoyl, cyano, amino, maleimide, formyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidoxycarbonyl, vinylsulfonyl, substituted or unsubstituted lower alkoxycarbonyloxy, or substituted or unsubstituted allyloxycarbonyloxy, can be produced by synthesizing Compounds (Idax) or Compounds (Idbx) in accordance with Process 4x, followed by the method described in Process 1x-1 through Process 1x-9.

Process 5x: Compounds where X^{1x} represents R^{8x}-C(=O)-O or O-C(=O)-R^{9x}

Of Compounds (Ix), those compounds (Iex) where X^{1x} represents R^{8x}-C(=O)-O (wherein $R^{8x}$is defined as described above) or O-C(=O)-R^{9x} (wherein $R^{9x}$is defined as described above) can be obtained by dehydration condensation for instance using a combination of polyalkylene glycol Ax and polycarboxylic acid, or a carboxylic acid derivative of polyalkylene glycol Ax and a polyol.

The dehydration condensation method may be a method of dehydration in the presence of an acidic or basic catalyst such as is used for standard ester synthesis, or may be a method of condensing corresponding alcohols and carboxylic acids using a condensing agent such as N,N'-dicyclohexylcarbodiimide or the like in an appropriate solvent such as methyl formamide, dimethyl sulfoxide, acetonitrile, pyridine, or methylene chloride or the like. Furthermore, the target substance can also be synthesized by the aforementioned process by reacting an acidic halogen compound and the corresponding alcohol.

The reaction conditions and purification conditions for each process shall conform to the previously mentioned processes.

Process 5x-1

Of Compounds (Iex), those compounds where R^{2x} is carboxy, carbamoyl, cyano, amino, maleimide, formyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidoxycarbonyl, vinylsulfonyl, substituted or unsubstituted lower alkoxycarbonyloxy, or substituted or unsubstituted allyloxycarbonyloxy, can be produced by synthesizing Compounds (Iex) in accordance with Process 5x, followed by the method described in Process 1x-1 through Process 1x-9.

Process 6x: Compounds where X^{1x} represents R^{6ax}-O-C(=O)-NH or R^{4x}-NH-C(=O)-O

Of Compounds (Ix), those compounds (Ifax) where X^{1x} represents R^{6ax}-O-C(=O)-NH (wherein R^{6ax} is defined as described above) can be produced by the following methods for instance.

Raw products containing compounds (Ifax) can be obtained by reacting three or more moles of a carbonate derivative of polyalkylene glycol Ax with a commercial polyamine or a polyamine produced from a polyol using a combination of the aforementioned methods. Incidentally, carbonate derivatives of polyalkylene glycol Ax can be produced by following the methods of Talia Miron et al. (Bioconjugate Chemistry, Volume 4, page 568 (1993)]. Furthermore, the carbonate derivative of polyalkylene glycol Ax may be N-hydroxysuccinimidyl carbonate, p-nitrophenyl carbonate, or an imidazolcarbonyloxy derivative or the like.

Of Compounds (Ix), those compounds (Ifbx) where X^{1x} represents R^{4x}-NH-C(=O)-O (wherein R^{4x} is defined as described above) can be produced by the following methods for instance.

Compounds (Ifbx) can be obtained by reacting a carbonate derivative of a polyol with an amino derivative of polyalkylene glycol Ax as described above.

Compounds (Ifax) and Compounds (Ifbx) can also be selectively produced by a combination of protecting the functional groups in accordance with other production methods and then removing the protection.

The reaction conditions and purification conditions for each process shall conform to the previously mentioned processes.

Process 6x-1

Of Compounds (Ifx), those compounds where R^{2x} is carboxy, carbamoyl, cyano, amino, maleimide, formyl, carbonyl halide, lower alkyl halide, isocyanate, isothiocyanate, succinimidoxycarbonyl, substituted or unsubstituted allyloxycarbonyl, benzotriazolyloxycarbonyl, phthalimidoxycarbonyl, vinylsulfonyl, substituted or unsubstituted lower alkoxycarbonyloxy, or substituted or unsubstituted allyloxycarbonyloxy, can be produced by synthesizing Compounds (Ifx) in accordance with Process 6x, followed by the methods described in Process 1x-1 through Process 1x-9.

A compound with three or more chains can also be acquired by bonding R^{1x}-$(Mx)_{nx}$-X^{1x} to Lx to make a single chain or double chain compound and then bonding an identical or different R^{1x}-$(Mx)_{nx}$-X^{1x} to Lx using the same reaction as described above. For instance, a compound with one or two chains can be obtained by bonding a polyalkylene glycol to functional groups in one or more locations on Lx using any of the reactions shown in the methods of Process 1x through Process 6x. The ratio of single chain and double chain compounds produced can be adjusted by changing the ratio of the polyalkylene glycol used in the reaction and the raw materials used to build the Lx component structure, and it is also possible to obtain product where single chain or double chain compound is the main component.

The single or double chain compound obtained may be used in the next step without modification, or may be purified to any level of purity corresponding to the number of branches of polyalkylene glycol or to a high purity, in accordance with the methods shown in Process 1x.

A compound with three or more chains can be produced by bonding the same or different polyalkylene glycol to the single or double chain compound obtained in this manner, using any of the methods shown in Process 1x through Process 6x. Incidentally, the third or higher polyalkylene glycol can be attached using the same reaction as the reaction used to obtain the single or double chain compound, but it is also acceptable to use a different reaction with a different bonding pattern. For instance, if a compound which has a plurality of functional groups such as hydroxyl groups, amino, or carboxy or the like is used as the raw material for building the Lx component structure, a single or double chain compound where X^{1x} represents O is first obtained by the method shown in Process 1x, and then a third or higher polyalkylene glycol can be reacted using a method shown in Process 4x such that X^{1x} is R^{4x}-NH-C(=O)-R^{5x}. As described above, a compound with three or more chains where a plurality of polyalkylene glycol is bonded to Lx in the same or different bonding patterns can be obtained by combining Process 1x through Process 6x. Furthermore, the polyalkylene glycol used at each reaction step may have different molecular weights, and the target substance can easily be obtained by using polyalkylene glycol units with different average molecular weights in the reaction where each of the polyalkylene glycol units is bonded to Lx.

Furthermore, in the reaction where polyalkylene glycol is introduced to Lx, it is also possible to leave one or more functional groups (for instance one or more hydroxyl groups for the case of Process 1x) on the Lx, and then react and bond a polyalkylene glycol after protecting the other functional groups with an appropriate protective group, and then later remove the protective group.

The polyalkylene glycol of the present invention can be obtained by the aforementioned production methods even if compounds other than those compounds specifically shown in the production method are used.

Incidentally, as described above, the polyalkylene glycol used as the raw material in the steps of Processes 1x through 6x may be a commercial product or may be easily produced using the various methods compiled by Samuel Zalipsky or the like [Bioconjugate Chemistry, Volume 6, page 150 (1995)].

The polyalkylene glycol obtained can be purified to branched polyalkylene glycol with any arbitrary level of purity according to the number of branches of polyalkylene glycol, using a method such as silica gel chromatography, reverse phase chromatography, hydrophobic chromatography, ion exchange chromatography, gel filter chromatography, recrystallization, or extraction or the like.

The branched polyalkylene glycol obtained can be bonded directly or through a spacer to the aforementioned amino acid side chain of a physiological polypeptide, an N terminal amino group, or a C terminal carboxyl group.

The spacer is preferably an amino acid or a peptide, but other options are possible so long as a polyalkylene glycol units can be bonded. The amino acid may be a natural amino acid such as lysine or cystine or the like, or may be ornithine, diamino propionic acid, or homocystein or the like. Cystine is more preferable. The peptide is preferably made from between 2 and 10 amino acid residues. In addition to amino acid or peptide, the spacer may be glycerol, ethylene glycol, or a saccharide or the like. Here, saccharide refers to monosaccharides or disaccharides such as glucose, galactose, sorbose, galactosamine, or lactose or the like.

These spacers form bonds such as amide bonds, thioether bonds, or ester bonds or the like with residue group side chains such as lysine, cystein, arginine, histidine, serine, and threonine or the like on a physiological polypeptide molecule, or amide bonds or ester bonds with the C terminal carboxyl group of the polypeptide, or amide bonds with the N terminal amino group of the peptide. These bonds can be made using normal peptide synthesis methods [Izumiya et al., *Pepuchido Gousei no Kisou to Jikken* (Peptide Synthesis Fundamentals and Experiments) (1985), Maruzen] or gene recombinant methods.

In this case, the amino acid or peptide or the like which will become the spacer is preferrably introduced to the C terminal carboxyl group at the same time that the physiological polypeptide is synthesized, but the spacer may also be bonded after the physiological polypeptide is synthesized.

Furthermore, the C terminal carboxyl group of the polypeptide can be chemosynthetically activated before bonding the spacer. Furthermore, a spacer which has been bonded beforehand to a polyalkylene glycol can be bonded to a physiological polypeptide using the aforementioned method.

The antibody used in the present invention may be obtained as a monoclonal antibody or a polyclonal antibody using commonly known methods [Antibodies – A Laboratory Manual, Cold Spring Harbor Laboratory (1988)].

Either polyclonal antibodies or monoclonal antibodies may be used as the antibodies used with the present invention, but monoclonal antibodies are preferable.

The monoclonal antibodies of the present invention may be hybridoma produced antibodies, humanized antibodies, or antibody fragments thereof or the like.

Human chimera antibodies or human CDR transplant antibodies or the like may be suggested as humanized antibodies.

Human chimera antibodies refers to antibodies comprising the heavy chain variable regions of non-human animal antibodies (hereinafter, heavy chain is referred to as H chain, variable region is referred to as V region, also referred to as HV or VH) as well as light chain variable regions (hereinafter, light chain is referred to as L chain, also referred to as LV or VL), and the heavy chain constant regions of human antibodies (hereinafter, constant region is referred to as C region, also referred to as CH) as well as the light chain constant regions (hereinafter also referred to as CL). Non-human animals include mice, rats, hamsters, and rabbits or the like, and if hybridoma cells can be produced, any type may be used.

Human CDR transplant antibodies refers to the CDR amino acid sequence of the V region of H chains or L chains of non-human antibodies which have been moved to an appropriate location in the V region of an H chain or L chain on a human antibody.

Antibody fragments may be Fab, Fab', $F(ab')_2$, single strand antibodies, disulfide stabilized V region fragments, and peptides containing complementary determining regions, or the like.

Fab are antigen bonding active fragments with a molecular weight of approximately 50,000, which are obtained by using papain enzyme to break apart the upper peptide region of 2 disulfide bonds which cross-link 2 H chains in the IgG hinge region,

and which are made from approximately half of the N terminal side of an H chain and all of an L chain.

Fab' are antigen bonding active fragments with a molecular weight of approximately 50,000 which are broken on at the disulfide bonds between the hinges of the aforementioned $F(ab')_2$.

$F(ab')_2$ are antigen bonding active fragments with a molecular weight of approximately 100,000, obtained by using trypsin enzyme to break apart the lower part of two disulfide bonds in the hinge region of IgG, and which are made by bonding 2 Fab regions in the hinge area.

Single strand antibody (hereinafter also referred to as scFv) refers to VH-P-VL or VL-P-VH polypeptides wherein a single VH and a single VL are connected using an appropriate peptide linker (hereinafter referred to as P). The VH and VL included in the scFv used in the present invention may be either a monoclonal antibody of the present invention or a human CDR transplant antibody.

Disulfide stabilized V region fragments (hereinafter also referred to as dsFv) refers to fragments wherein polypeptides, where one amino acid residue group from each of VH and VL have been substituted by a cystine residue group, are bonded with a disulfide bond. The amino acid residue groups which are replaced by the cystine residue groups can be selected based on the predicted three-dimensional structure of the antibody using the methods disclosed by Reiter et al. [Protein Engineering, Volume 7, page 697 (1994)]. The VH and VL included in the disulfide stabilized antibody of the present invention may be either a monoclonal antibody or a human CDR transplant antibody.

The chemical modified polypeptide used with the present invention is preferably a chemical modified polypeptide which chemically modifies interferon, and medical drugs which contain these chemical modified polypeptides are also preferable. Furthermore, medical drugs which contain chemical modified polypeptides which chemically modify interferon include drugs for treating multiple sclerosis, drugs for treating hepatitis, drugs for treating diseases related to vascularization, drugs for treating malignant tumors, drugs for treating eye disease, and drugs for treating skin disease, or the like, but drugs for treating multiple sclerosis are preferable.

Ointments which contain chemical modified polypeptides of the present invention can be produced by standard ointment production methods. For instance, the ointment base may be a hydrophilic base such as macrogol or solbase which can be used to prepare ointments of water-soluble drugs, emulsion bases such as hydrophilic ointment, hydrophilic Vaseline, hydrophilic plastibase, or purified lanolin or the like, or non-fatty bases such as bentonite, veegum, starch paste, or sodium alginate or the like, and these ointments are produced by mixing chemically modified polypeptide aqueous solution, suspension solution, or freeze-dried powder thereof into these ointment bases at a weight ratio of between 0.0000001 and 10%, in accordance with standard ointment preparation methods disclosed in documentation or the like [*Yakuzaigaku* (Pharmacology) (Sezaki et al.) Published in 1992, Yokogawa Shoten].

Common additives which are normally used in these forms may also be used, including buffer agents, fillers, pH adjusting agents, stabilizers, preservatives, moisturizers, emulsifiers, lubricants, sweeteners, colorants, and antioxidants or the like.

The administration method may be a method where the ointment of the present invention is applied transdermally or transmucosally, or by another allowed method, and ingredients suitable for the administration method may also be used. Furthermore, the quantity of chemically modified polypeptide in the ointment may vary depending on conditions such as the type of the disease and the condition of the patient, but the physiological polypeptide in the chemically modified polypeptide is preferably included at a weight ratio of between 0.0000001% and 10% in 1 g of ointment.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the stabilizing effect of chemically modified recombinant human interferon-β in hydrophilic ointment. In Fig. 1, the symbols (−◆−, −■−) have the following meanings.

−◆− : Change in the residual amount of rhIFN-β in unmodified rhIFN-β

−■− : Change in residual amount of rhIFN-β in 5CHTM(2EA)-rhIFN-β

Fig. 2 shows the stabilizing effect in macrogol ointment of chemically modified bovine Cu, Zn type superoxide dismutase. In Fig. 2, the symbols (−△−, −○−, −□−, −●−) have the following meanings.

−△− : Macrogol ointment containing chemically modified bSOD (5CHTM(sEA)-bSOD

−○− : Ointment containing bSOD macrogol

−□− : Ointment containing macrogol where bSOD and mPEG are mixed

—●— : bSOD aqueous solution

Fig. 3 shows the stabilizing effect (SDS-PAGE) of recombinant human granulocyte colony stimulating factor derivative in macrogol ointment. In Fig. 3, the symbols ((1), (2), (3), (4), (5), (6), (7), (8)) have the meanings shown below. The vertical axis shows the molecular weight.
(1): Molecular weight marker
(2): G-CSF derivative aqueous solution
(3): Immediately after ointment preparation
(4): After 1 hour at room temperature
(5): After 1 day at room temperature
(6): After 2 days at room temperature
(7): After 3 days at room temperature
(8): After 7 days at room temperature

Fig. 4 shows the stabilizing effect (SDS-PAGE) of chemically modified recombinant human granulocyte colony stimulating factor derivative in macrogol ointment. In Fig. 4, the symbols ((1), (2), (3), (4), (5), (6), (7), (8)) have the meanings shown below. The vertical axis shows the molecular weight.
(1): Molecular weight marker
(2): Chemically modified G-CSF derivative aqueous solution
(3): Immediately after ointment preparation
(4): After 1 hour at room temperature
(5): After 1 day at room temperature
(6): After 2 days at room temperature
(7): After 3 days at room temperature
(8): After 7 days at room temperature

Fig. 5 shows the stabilizing effect (SDS-PAGE) at 37°C of recombinant human granulocyte colony stimulating factor derivative in hydrophilic ointment. In Fig. 5, the symbols ((1), (2), (3), (4), (5), (6), (7), (8), (9)) have the meanings shown below. The vertical axis shows the molecular weight.

(1): Molecular weight marker
(2): G-CSF derivative aqueous solution
(3): Immediately after ointment preparation
(4): After 30 minutes at 37°C
(5): After 1 hour at 37°C
(6): After 2 hours at 37°C
(7): After 4 hours at 37°C
(8): After 8 hours at 37°C
(9): After 24 hours at 37°C

Fig. 6 shows the stabilizing effect (SDS-PAGE) at 37°C of chemically modified recombinant human granulocyte colony stimulating factor derivative in hydrophilic ointment. In Fig. 6, the symbols ((1), (2), (3), (4), (5), (6), (7), (8), (9)) have the meanings shown below. The vertical axis shows the molecular weight.
(1): Molecular weight marker
(2): Chemically modified G-CSF derivative aqueous solution
(3): Immediately after ointment preparation
(4): After 30 minutes at 37°C
(5): After 1 hour at 37°C
(6): After 2 hours at 37°C
(7): After 4 hours at 37°C
(8): After 8 hours at 37°C
(9): After 24 hours at 37°C

Fig. 7 shows the stabilizing effect (SDS-PAGE) at room temperature of recombinant human granulocyte colony stimulating factor derivative in hydrophilic ointment. In Fig. 7, the symbols ((1), (2), (3), (4), (5), (6)) have the meanings shown below. The vertical axis shows the molecular weight.

(1): Molecular weight marker
(2): G-CSF derivative aqueous solution
(3): Immediately after ointment preparation
(4): After 10 days at room temperature
(5): After 20 days at room temperature
(6): After 30 days at room temperature

Fig. 8 shows the stabilizing effect (SDS-PAGE) at room temperature of chemically modified recombinant human granulocyte colony stimulating factor derivative in hydrophilic ointment. In Fig. 8, the symbols ((1), (2), (3), (4), (5), (6)) have the meanings shown below. The vertical axis shows the molecular weight.

(1): Molecular weight marker
(2): Chemically modified G-CSF derivative aqueous solution
(3): Immediately after ointment preparation
(4): After 10 days at room temperature
(5): After 20 days at room temperature
(6): After 30 days at room temperature

BEST FORM FOR CARRYING OUT THE INVENTION

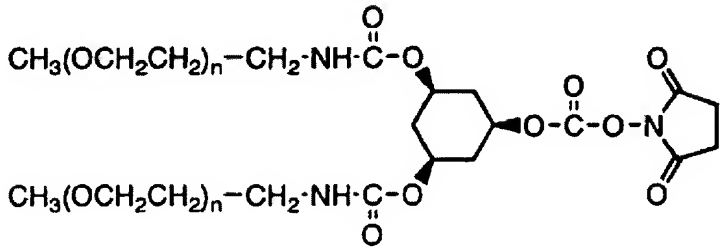
The following examples described the present invention in detail, and are not to be interpreted as a restriction on the scope of the invention. Abbreviations in the examples have the following meaning unless otherwise stated. Incidentally, the symbols for the amino acids and the protective groups thereof which are used in this specification conform to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [European Journal of Biochemistry, Volume 138, page 9 (1984)].

HPLC: High-performance Liquid Chromatography
MALDI-TOF MS: Matrix Assisted Laser Desorption Ionization Time of Flight Mass
FAB MS: Fast Atom Bombered Mass
UV: Ultraviolet

RI: Refractive Index
NMR: Nuclear Magnetic Resonance
ELISA: Enzyme-linked Immunosorbent Assay
SDS-PAGE: Sodium Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis
PEG: Polyethylene glycol
mPEG: Monomethoxy polyethylene glycol
INF: Interferon
hINF: Human interferon
rhIFN: Recombinant human interferon
G-CSF: Granulocyte colony stimulating factor
rhG-CSF: Recombinant human granulocyte colony stimulating factor
SOD: Superoxide dismutase
bSOD: Bovine superoxide dismutase
hSOD: Human superoxide dismutase
DSC: N,N'-disuccinimidyl carbonate
TEA: Triethylamine
DMF: N,N-dimethylformamide
DMSO: dimethylsulfoxide
NHS: N-hydroxysuccinimide
Ts: p-toluenesulfonyl
TsCl: p-toluenesulfonyl chloride
DMAP: dimethylaminopyridine
PyBOP: benzotriazole-1-yloxytripyridinophosphonium hexafluorophosphate
HOBt: N-hydroxybenzotriazole
DCC: N,N'-dicyclohexylcarbodimide

LAH: Lithium aluminum hydride
NMM: N-methylmorpholine
TFA: Trifluoroacetic acid
CDI: N,N'-carbonyldiimidazole

Example 1: Synthesis of Branched Polyethylene Glycol Cyclohexane Derivative with Two 5kDa Chains
Symbol: 5CHTO(2UU)
Structure:



420.5 mg (2.5 mmol) of cis,cis-1,3,5-cyclohexanetriol dihydrate (produced by Fluka Co., Ltd) and 3.2 g (12.5 mmol) of DSC were dissolved in 20 mL of acetonitrile in an argon environment, 2.1 mL (12.5 mmol) of TEA were added, and the solution was mixed for one day and night at room temperature. The solvent was removed under reduced pressure, and then extraction was performed by adding chloroform and 0.1 mol/L HCl. After drying the chloroform layer using sodium sulfate anhydride, the solvent was removed at reduced pressure, and 357 mg of cis,cis-1,3,5-tris(succcinimidyloxycarbonyloxy) cyclohexane was produced (yield: 25.7%).

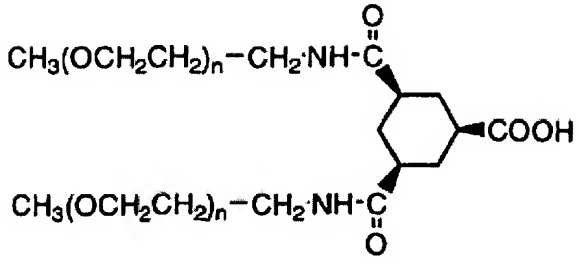
Next, 500 mg (0.1 mmol) of monomethoxypolyethylene glycol propylamine (mPEG-NH_2) (average molecular weight 5000, produced by Nippon Oil and Fats, Ltd.) and said cis,cis-1,3,5-tris(succcinimidyloxycarbonyloxy) cyclohexane were dissolved in 12.5 mL of methylene chloride, 28 μL of TEA were added, and the solution was mixed at room temperature for 2 hours.

Next, the reaction liquid was dripped into diethyl ether, the white precipitate produced was dried at reduced pressure, and 472 mg of residue was obtained (yield 94.4%). 372 mg of this substance was purified using reverse phase HPLC. The column was a TSK gel ODS120-T (30 mm x 250 mm) (Tosoh), and using 0.1% TFA aqueous solution as the mobile phase, elution was performed at a flowrate of 10 mL/min using the straight-line concentration curve of acetonitrile between 0 and 90%. 30 mL of the target fraction with an average molecular weight of 10,000 was recovered, the acetonitrile was removed under reduced pressure, and extraction was performed using chloroform. This was dripped into diethyl ether, and the white precipitate was collected and dried at reduced pressure to obtain 121.7 mg of the target substance (yield 32.7%)

<Gel Suspension HPLC Analysis>
Mobile phase: 150 mmol/L of sodium chloride, 20 mmol/L sodium acetate buffer solution (pH 4.5)
Flowrate: 0.7 mL/min
Detection: RI
Separation column: TSK gel G-2000SW_{XL} (7.8 x 300 mm) (Tosoh)
Column temperature: Room temperature
Retention time: 12.2 minutes

<^{1}H-NMR Analysis ($CDCl_3$, 300 MHz)>
δ (ppm): 3.61 (s, 8nH), 3.41 (s, 6H), 4.69 (br, 4H), 1.77 (brm, 4H), 5.30 (br, 2H), 0.8-3.4 (m, 9H), 2.84 (s,4H)

Example 2: Synthesis of Branched Polyethylene Glycol Cyclohexane Derivative with Two 5 kDa Chains
Symbol: 5CHTC(2AA)
Structure:



84.0 mg (0.388 mmol) of cis,cis-1,3,5-cyclohexane tricarboxylic acid (produced by Fluka Co., Ltd) were dissolved in 50 mL of DMF, 270.2 mg (2.0 mmol) of HOBt and 1.04 g (2.0 mmol) of PyBOP were added, and the solution was mixed for 30 minutes at 0°C. Next, 5 g (1.0 mmol) of monomethoxypolyethylene glycol propylamine (mPEG-NH2) (average molecular weight 5000, produced by Nippon Oil and Fats, Ltd.) and 219 .7 µL of NMM were added, and the solution was stirred for one day and night. The pH was adjusted using 1 mol/L HCl, and then extraction was performed using chloroform. After drying the organic layer with anhydrous sodium sulfate, the solution was dripped into diethyl ether. A white precipitate was collected and 3.78 g (Yield 75.6%) of raw reaction products containing the target compound was obtained. Next, the product was purified using a 300 mL of DEAE-Sepharose F. F. column (Amersham-Pharmacia Biotec Corp.). The raw reaction product was dissolved in water and inserted into the column, and after washing the column with 600 mL of water, elution was performed using between 0.6 and 1.2 mmol/L of sodium chloride aqueous solution. Next, the target fraction was extracted using chloroform, and after removing the solvent under reduced pressure, 610.4 mg (yield 65.2%) of the target substance was obtained.

<Gel Filter HPLC Analysis>

The same analysis as Example 1 was performed using a TSK gel G-2000SW_{XL} column.

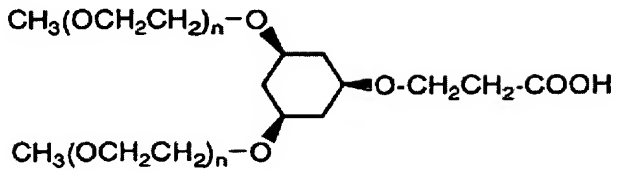
Retention time: 12.0 minutes

<^{1}H-NMR Analysis ($CDCl_3$, 300 MHz)>

δ (ppm): 1.56 (m, 3H), 2.1-2.5 (m, 6H), 1.77 (m, 4H), 2.1-2.3 (br, 4H), 3.38 (br, 4H), 3.64 (s, 8nH), 3.36 (s, 6H), 6.46 (t, J=5.23 Hz, 2H)

Example 3: Synthesis of Branched Polyethylene Glycol Cyclohexane Derivative with Two 5 kDa Chains

Symbol: 5CHTO(2EA)
Structure:



50 g (10 mmol) of mPEG [average molecular weight 5000, produced by Nihon Oil and Fats, Ltd.] was dissolved in 150 mL of toluene, and then dehyration reflux was performed. 3.5 mL (25 mmol) of TEA was added by dripping over 1 hour, and then a solution where thionyl bromide (1.55 mL) was dissolved in toluene (13.6 mL) was added by dripping. After refluxing for 1 hour, filtering was performed using celite, and then allowed to set for 4 hours at room temperature. Next, the solution was heated to 50°C, and 5 g of activated carbon was added. The activated carbon was removed using celite, and then the solution was allowed to sit at 4°C for one day and night. The next day, the supernatant was removed, and the residue was dissolved in 250 mL of ethanol at 60°C. Next, 3 g of activated carbon was added and after removing the activated carbon with celite, the solution was allowed to sit at 4°C for one day and night. The next day, the residue was washed with cold ethanol and diethyl ether, and dried, and 32.87 g (yield 65.74%) of bromated mPEG (mPEG-Br) was obtained.

<^{1}H-NMR Analysis ($CDCl_3$, 300 MHz)>
δ (ppm): 3.64 (s, 4nH), 3.38 (s, 3H), 3.48 (t, J= 6.3 Hz, 2H), 3.1 (t, J=6.3 Hz, 2H)

After sufficiently drying, 1.322 g (10 mmol) of cis,cis-1,3,5-cyclehexanetriol dihydrate was dissolved in 25 mL of anhydrous DMF and then added by dripping into 0.48 g (11 mmol) of sodium hydroxide in an argon environment, and then stirred for 30 minutes. 10 g (2 mmol) of the aforementioned mPEG-Br dissolved in 25 mL of DMF was added by dripping, and the solution was stirred at room temperature for one day and night. Next, the reaction liquid was dripped into diethyl ether, and the white precipitate was dried at reduced pressure. Next, the dried powder was dissolved in an appropriate quantity of water, and the pH was adjusted to 3 using 1 mol/L of HCl, and then extraction was performed using chloroform. The organic layer was dried using anhydrous sodium sulfate, and then the solvent was removed by reduced pressure. The residue was dissolved in a small amount of methylene chloride, then dripped into diethyl ether, and the precipitate performed was dried under reduced pressure. 7.5 g (yield 75.0%) of a single chain raw reaction product with a single molecule of mPEG bonded to cyclehexanetriol was obtained.

50 mL of toluene was added to 5 g of the raw reaction product obtained and dehydration reflux was performed for one day and night. Furthermore, 5.5 g of mPEG-Br (1.1 mmol) was dissolved in 50 mL of toluene, and dehydration reflux was performed at 160°C for one day and night. Next, 144 mg (3.3 mmol) of sodium hydroxide was added to the above toluene solution of raw reaction product, and after stirring for 30 minutes, the mPEG-Br toluene solution was added by dripping. After dehydration reflux was performed for one day and night, the insolubles were removed by filtering, and then dried under reduced pressure. The pH was adjusted to between 1 and 2, by adding 1 mol/L of HCl, and then extraction was performed using chloroform. The organic layer was dried using anhydrous sodium sulfate, the solvent was removed under reduced pressure, the residue was dissolved by adding a small amount of methylene chloride, and then the solution was dripped into diethyl ether The white precipitate produced was vacuum dried, and 7.73 g (yield 73.6%) of raw reaction product containing the target compound was obtained.

1.5 g of the raw reaction product was dissolved in an 8% aqueous solution of potassium hydroxide, 150 mg (2.11 mmol) of acrylamide was added, and the solution was stirred for 7 hours at room temperature. An additional 150 mg (2.11 mmol) of acrylamide was added, and the solution was stirred for 4 days at room temperature. The pH of the reaction liquid was adjusted to 3 using 1 mol/L HCl, extraction was performed using chloroform, the organic layer was dried using anhydrous sodium sulfate, and then the solvent was removed by reduced pressure. The residue was dissolved in methylene chloride, dripped into diethyl ether, the precipitate performed was removed by filtering, and then vacuum dried to obtain 1.017 g (67.8%) of the raw target substance.

The substance was inserted into a 60 mL DEAE-Sepharose F. F. column (Amersham-Pharmacia Biotech), and elution was performed using between 0.4 and 1.4 mmol/L sodium chloride aqueous solution. The fraction containing the target substance was extracted using chloroform. The chloroform layer was dried using anhydrous sodium sulfate, and then the solvent was removed by reduced pressure to obtain 52 mg of the target substance.

<Gel Filter HPLC Analysis>

A measurement was made under the same conditions as Example 1 using a TSK gel G-2000SW_{XL} column.

Retention time: 12.7 min

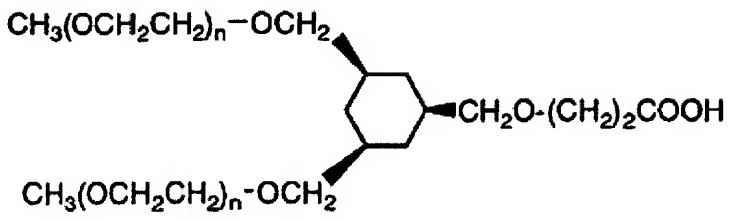
<^{1}H-NMR Analysis ($CDCl_3$, 300 MHz)>

δ (ppm): 2.59 (t, J= 16.0 Hz, 2H), 0.8-3.4 (m, 9H), 3.64 (s, 8nH), 3.38 (s, 6H)

Example 4: 5kDa Double-branched Polyethylene Glycol Cyclohexane Derivative Compound

Abbreviation: 5CHTM(2EA)

Structure:



400 g (80 mmol) of mPEG (average molecular weight 5000, Nippon Oil and Fats, Ltd.) was dissolved in 1L of toluene and methylene chloride. 50 g of TsCl was added, followed by 46.4 mL of TEA, and the result was stirred at room temperature for 8 hours. Next, 50 g of TsCL was added and the mixture was stirred for 16 hours. Insoluble material was separated by filtration and the solvent was condensed under reduced pressure. The resulting residue was dissolved in a small amount of chloroform and dripped in diethyl ether. The generated white precipitate was recovered and dried under reduced pressure to obtain 344 g of tosyl ester mPEG (mPEG-Ots) (yield: 86.0%).

<^{1}H-NMR Analysis ($CDCl_3$, 300 MHz)>
δ (ppm): 2.45 (s, 3H), 3.38 (s, 3H), 3,70 (s, 4nH), 4.16 (t, J=5.0Hz, 2H), 7.34 (d, J=6.8Hz, 2H), 7.80 (d, J=8.1Hz, 2H)

Following dissolution of 1 L of DMF in 344 g of mPEG-OTs, 54 g of sodium iodide was added, and the solution stirred for 1 hour at 80 to 90°C. The insoluble material was separated by filtration and the filtered solution was added to diethyl ether by dripping. The resulting white precipitate was collected by filtration and dried under reduced pressure. The residue was dissolved in 1.5 L of a 10% sodium thiosulfate solution and stirred for some time, and then extracted using chloroform. The solvent was removed under reduced pressure to obtain 314 g of iodized mPEG (mPEG-I) (yield: 78.5%).

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 3.27(t, J=6.9Hz, 2H), 3.38(s, 3H), 3.67(s, 4nH)

40 g of cis,cis-1,3,5-cyclohexane tricarboxylic acid (Fluka, Inc.)was dissolved in 1 L of n-propanol and 20 mL of concentrated sulfuric acid and stirred for 72 hours at room temperature. Thereafter, an appropriate amount of ethyl acetate was added to the reaction solution, undergoing continuous neutralization with a saturated sodium hydrogen carbonate solution. The reaction solution was extracted with ethyl acetate and the organic layer was dried with anhydrous sodium sulfate. The solvent was removed under reduced pressure to obtain 72.4 g of cis,cis-1,3,5-cyclohexane tricarboxylic acid apropyl ester (yield: quantitative).

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >
δ (ppm): 0.94(t, J=6.4Hz, 9H), 1.65(m, 6H), 4.05(t, J=6.6Hz, 6H), 1.56, 2.25, 2.40 (value of each m, 9H)

1.19g of LAH was dissolved in 50 mL of diethyl ester, following which 12.5 mL of the diethyl ester containing 3.2 g of cis,cis-1,3,5-cyclohexane tricarboxylic acid n-propyl ester was added by dripping, in an argon atmosphere. The mixture was refluxed for 41 hours while being stirred. Next, 2.5 mL of water was added by dripping and the solution was stirred similarly for 15 minutes. After that, 5 mL of ethanol was added by dripping and the result was stirred for 3 hours at room temperature. The reaction solution was filtered and insoluble material was extracted with boiling ethanol. This ethanol solution was combined with the previous filtered solution and the solvent was removed under reduced pressure. The obtained residue was extracted with boiling 1,4-dioxane and dried with sodium sulfate. The solvent was removed under reduced pressure to obtain 1.50 g of cis,cis-1,3,5-cyclohexane trimethanol (yield: 91.7%).

< Mass Analysis (FAB-MS) >

Actual Value: $(M + H)^+$ 175

Theoretical Value: $C_9H_{18}O_3$ = 174

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >
δ (ppm): 3.21(t, J=5.9Hz, 6H), 4.35(t, J=5.1Hz, 3H), 0.43, 1.40, 1.75 (value of each m, 9H)

2.5 g (14 mmol) of cis,cis-1,3,5-cyclohexane trimethanol was dissolved in 10 mL of anhydrous DMF, and then 2.28 g (46.2 mmol) of sodium hydride was added in an argon atmosphere at 0°C and was stirred for 30 minutes.

40 g (8 mmol) of the dissolved mPEG-I was added to 50 mL of the DMF solution by dripping and was stirred for one day and night at room temperature. Then, the reaction solution was added to diethyl ether by dripping and the resulting precipitate was dried under reduced pressure. The dried precipitate was then dissolved in an appropriate amount of water, whereupon the solution was adjusted to a pH of 3 with 1 mol/L hydrochloric acid and extracted with chloroform, following which the organic layer was dried with anhydrous sodium sulfate and the solvent removed under reduced pressure. The residue was dissolved in a small amount of methylene chloride, and then added to diethyl ether by dripping, following which the resulting precipitate was dried under reduced pressure to obtain 33.0 g (83%) of a double-chain mPEG raw reaction product with two molecules bound to cis,cis-1,3,5-cyclohexanetrimethanol.

14.0 g of raw reaction product was dissolved in an 8% solution of potassium hydroxide, and 1.8 g (16.7 mmol) of acrylamide was added and the mixture stirred for 7 hours at room temperature. Another 1.8 g (16.7 mmol) of acrylamide was added and the mixture was stirred for 4 days at room temperature. The reaction solution was adjusted to a pH of 3 with 1 mol/L hydrochloric acid, the mixture was then extracted and after the organic layer was dried with anhydrous sodium sulfate, the solvent was removed under reduced pressure. Following the dissolution of the residue in methylene chloride, the solution was added to diethylene ether by dripping, and the resulting precipitate was filtered and dried under reduced pressure to obtain 10.2 g (73%) of raw reaction product. The raw reaction product was purified with a 1000 mL DEAE-Sepharose F.F column (Amersham-Pharmacia Biotech, Inc.). The elution was prepared and eluted with between 0.4 and 100 mmol/L of a sodium chloride solution. The target fraction was extracted with chloroform, the solvent removed from the chloroform layer under reduced pressure, and the residue was precipitated out using diethyl ether to obtain 500 mg of the target product.

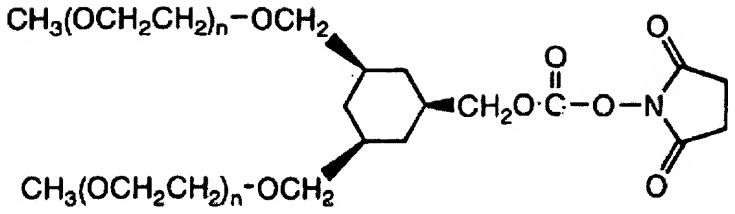
< Gel Filtration HPLC Analysis >

Analysis was conducted using a TSK gel G-2000SW_{XL} column in a manner similar to Example 1.

Retention time: 12.7 minutes
< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >
δ (ppm): 3.38(s, 6H), 3.64(s,8nH), 0.85, 1.26 (each m value, 9H)

Example 5 5kDa Double-branched Polyethylene Glycol Cyclohexane Derivative Compound

Structure:



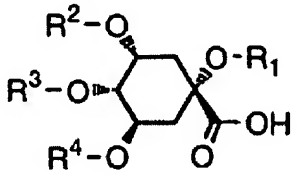
Similar to Example 4, a double-chain mPEG raw reaction product with two molecules bound to cis,cis-1,3,5-cyclohexane trimethanol was obtained. The TSK gel ODS120-T column shown in Example 1 was used to purify 2.7 g of the raw reaction product by reverse phase HPLC whereby the fraction containing only the two-chain PEG derivative was recovered. From this fraction, acetonitrile was removed under reduced pressure and the mixture extracted with chloroform. The chloroform layer was dried with anhydrous sodium sulfate to obtain 227 mg of the double-branched PEG dried under reduced pressure (8.4% yield from raw reaction product). 1.2 mg (10 μmol) of DAP and 2.6 mg (10 μmol) of DSC were added to 20 mg (2 μmol) of the double-branched PEG derivative dried under reduced pressure, and 1 mL of methylene chloride was added, after which the solution was stirred for 6 hours under an argon stream at room temperature. The reaction solution was filtered and added to diethyl ether by dripping. The resulting precipitate was recovered and dried under reduced pressure to obtain 15 mg of the target product (yield: 75%).

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >
δ (ppm): 0.5-2.0(m, 9H), 2.84(s, 4H), 3.61(s, 8nH), 3.41(s, 6H)

Example 6: 5kDa Double-branched Polyethylene Glycol Cyclohexane Derivative Compound

Abbreviation: 5QNA(2UA), 5QNA(3UA), 5QNA(4UA)

Structure:



R^1, R^2, R^3, R^4=
$CH_3(OCH_2CH_2)_n$-NH-CO- and H

(In the formula of compound 5QNA(2UA) two of R^1, R^2, R^3, and R^4 are

$CH_3(OCH_2CH_2)_n$-NH-CO- and the remaining two are hydrogen atoms. In compound 5QNA(3UA) three of R^1, R^2, R^3, and R^4 are $CH_3(OCH_2CH_2)_n$-NH-CO- and the remaining R is a hydrogen atom. In compound 5QNA(4 3UA) all four R^1, R^2, R^3, and R^4 are $CH_3(OCH_2CH_2)_n$-NH-CO-).

3 mg of quinic acid ((1R, 3R, 4R, 5R)-(-)-quinic acid) was dissolved in 250 µL of DMF, and 17 µL of triethylamine and a catalytic amount of copper chloride were added thereto. To the mixture was added 344 mg of mPEG-NCO (molecular weight: 5,000, Shearwater Polymers, Inc., Structure: $CH_3(OCH_2CH_2)_n$-N=C=O), after which the mixture was stirred for 1 hour at room temperature. The reaction mixture was added by dripping to a ten-fold quantity of diethyl ether, and the resulting precipitate was recovered by filtration and dried under reduced pressure to obtain 306 mg (88%) of raw target product. The product was purified using DEAE Sepharose F.F. column (Amersham-Pharmacia Biotech, Inc.) in a manner similar to that in Example 2. The target fraction was extracted with chloroform, and the solvent was removed under reduced pressure to obtain the following compounds.

The yield of compound 5QNA(2UA) was 36 mg (yield: 10.5%) and the retention time was 2.4 minutes with gel filtration HPLC. The yield of compound 5QNA(3UA) was 24 mg (yield: 10.2%) and the retention time was 11.7 minutes with gel filtration HPLC . The yield of compound 5QNA(4UA) was 17 mg (yield: 5.4%) and the retention time was 11.1 minutes with gel filtration HPLC. Measurement was carried out using TSK gel $G2000SW_{XL}$ column under conditions similar to those in Example 1.

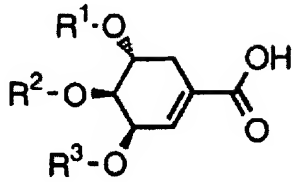
< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 5.7-4.8(m, 3H), 3.33(s, 6H, 9H, or 12H), 3.64(s, 8nH, 12nH or 16nH)

Example 7: 5kDa Double-branched Polyethylene Glycol Cyclohexane Derivative Compound

Abbreviation: 5SKA(2UA), 5SKA(3UA)

Structure:



R^1, R^2, R^3=
$CH_3(OCH_2CH_2)_n$-NH-CO- or H

(In the formula of compound 5SKA(2UA), two of R^1, R^2, and R^3 are $CH_3(OCH_2CH_2)_n$-NH-CO- and the remaining R is a hydrogen atom. In compound 5SKA(3UA) all three R^1, R^2, and R^3 are $CH_3(OCH_2CH_2)_n$-NH-CO-).

3.2 mg of shikimic acid was dissolved in 250 µL of DMF, and 15 µL of triethylamine and a catalytic amount of copper chloride were added. 300 mg of PEG-NCO (average molecular weight: 5,000, Shearwater Polymers, Inc., Structure: $CH_3(OCH_2CH_2)_n$-N=C=O) was added to the mixture and the mixture was stirred for 1 hour at room temperature. The reaction mixture was added to a ten-fold quantity of diethyl ether by dripping, and the resulting precipitate was recovered by filtration and dried under reduced pressure to obtain 270 mg (89%) of a raw target product. The product was purified using DEAE Sepharose F.F. column (Amersham-Pharmacia Biotech, Inc.) in a manner similar to that in Example 2. The target fraction was extracted with chloroform, and the solvent was removed under reduced pressure to obtain the following compounds.

The yield of compound 5SKA(2UA) was 4 mg (yield: 1.3%) and the retention time was 12.4 minutes with gel filtration HPLC. The yield of compound 5SKA(3UA) was 18 mg (yield: 6.0%) and the retention time was 11.7 minutes with gel filtration HPLC . Measurement was carried out using a TSK gel $G2000SW_{XL}$ column under conditions similar to those in Example 1.

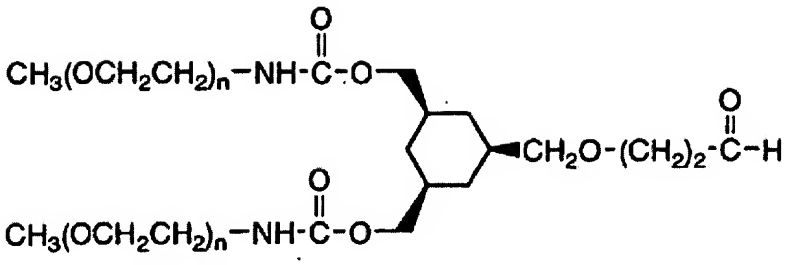
< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 6.6-5.1(m, 4H), 3.33(s, 6H or 9H), 3.64(s, 8nH or12nH)

Example 8: 5kDa Double-branched Polyethylene Glycol Cyclohexane Derivative Compound

Abbreviation: 5CHTM(2URa)

Structure:



50 mg of cis,cis-1,3,5-cyclohexane trimethanol synthesized in a manner similar to that in Example 4 was dissolved in 0.5 mL of dry DMF, following which17 mg of sodium hydride was added followed by stirring for 15 minutes at 0°C. 47 µL of 3-bromopropionaldehyde dimethylacetal was then added to the mixture, after which it was stirred for 16 hours at room temperature. It was then purified using a silica gel column to obtain 15 mL of a compound wherein propionaldehyde dimethylacetal was bound to cis,cis-1,3,5-cyclohexane trimethanol at position 1 (yield: 38%).

< ^{1}H-NMR Analysis (DMSO-d6, 300MHz) >
δ (ppm): 0.62(m, 9H), 1.54-1.88(m, 9H), 1.83(q, J=6.20Hz, 2H), 3.27(d, J=6.30Hz, 2H), 3.33(s, 6H), 3.39(d, J=6.30Hz, 4H), 3.46(t, J=6.20Hz, 2H), 4.51(t, J=5.70Hz, 1H)

< Mass Analysis (FAB-MS) >
Actual Value: $(M + H)^+$ 277
Theoretical Value: $C_{14}H_{28}O_5$ = 276

15 mg of the obtained compound was dissolved in 1 mL of dry DMF and 31 µL of triethylamine and a catalytic amount of copper chloride were added. 598 mg of mPEG-NCO (molecular weight: 5,000, Shearwater Polymers, Inc., structure: $CH_3(OCH_2CH_2)_n$-N=C=O) was added to the mixture, after which the mixture was stirred for 2 hours at room temperature. The reaction mixture was added to a ten-fold quantity of diethyl ether by dripping, and the resulting white precipitate was recovered by filtration and dried under reduced pressure. In a manner similar to that of Example 1, 578 mg of the obtained white solid was purified using reverse phase HPLC to obtain 383 mg. 100 mg of purified product was dissolved in a 70% acetic acid solution and stirred for 16 hours at 40°C. The reaction solution was neutralized with a saturated sodium bicarbonate solution and the mixture was extracted with chloroform. The reaction solution was concentrated under reduced pressure after being dried with anhydrous sodium sulfate. The solution was added to diethyl ether by dripping and the resulting white precipitate was recovered by means of filtration and then dried under reduced pressure.

The precipitate was purified a second time using reverse phase HPLC to obtain 39 mg (yield: 41%) of the target product.

< Gel Filtration HPLC Analysis >

Measurement was carried out using TSK gel G2000SW_{XL} column under conditions similar to those in Example 1.

Retention time: 12.7 minutes

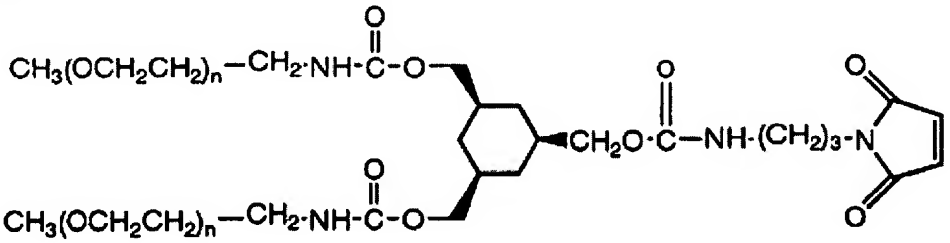
< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 3.38(s, 6H), 9.79(t, J=1.56Hz, 1H), 3.64(s, 8nH)

Example 9: 5kDa Double-branched Polyethylene Glycol Cyclohexane Derivative Compound

Abbreviation: 5CHTM(2UM)

Structure:



100 mg of cis,cis-1,3,5-cyclohexane trimethanol synthesized in a manner similar to that in Example 4 and 735 mg of DSC were dissolved in approximately 10 mL of acetonitrile, and 210 mg of DMAP was added and the solution stirred for 5 hours at room temperature. The solvent was removed under reduced pressure and extracted by the addition of methylene chloride and 0.1mol/L hydrochloric acid. The organic layer was removed under reduced pressure to obtain 333 mg of cis,cis-1,3,5-tris (succinimidyl oxycarbonyl oxymethyl)cyclohexane (yield: 97%).

FAB-MS: 598 $(M + H)^+$

30 mg (0.05 mmol) of the obtained compound and 500 mg (0.1 mmol) of mPEG-NH_2 (average molecular weight: 5,000, Nippon Oil and Fats, Ltd.) were dissolved in methylene chloride, 20 μL of TEA was added, and the mixture was stirred for 2 hours at room temperature.

Next, 42 μL (0.5 mmol) of propylene diamine (Aldrich, Inc.) was added and the mixture was stirred for another 2 hours at room temperature. The reaction solution filtrate was added to diethyl ether by dripping to obtain 430 mg (yield: 86%) of a powder from the resulting precipitate dried under reduced pressure.

425 mg of the obtained powder was dissolved in 200 mL of water, purified with a 20 mL SP Sepharose F.F. column (Amersham-Pharmacia Biotech, Inc.), and the target fraction containing the double-branched PEG was extracted with chloroform. The organic layer obtained was added to diethyl ether by dripping, and the purified precipitate was dried under reduced pressure. 62.5 mL (6.25 μmol) of obtained precipitate was dissolved in 0.5 mL of saturated sodium bicarbonate, and 2.1 mg of ice-cooled ethoxycarbonyl maleimide was added and stirred for 10 minutes. Next, 1.5 mL of water was added and the mixture was stirred for 15 minutes then extracted three times using chloroform. The chloroform layer was dried with anhydrous sodium sulfate and the solvent removed under reduced pressure to obtain the 25 mg of desired compound (yield: 40%).

< Gel Filtration HPLC Analysis >
Analysis was conducted using TSK gel G-2000SW_{XL} column under conditions similar to those in Example 1.

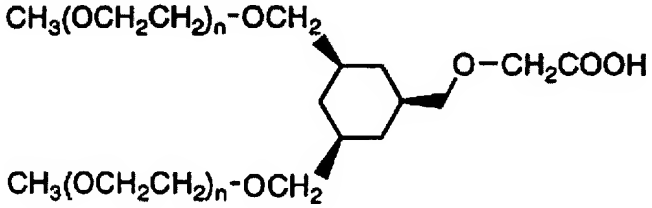
Retention time: 12.4 minutes

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >
δ (ppm): 0.63-0.75(m, 3H), 1.75-1.78(m, 12H), 3.1-3.3(m, 12H), 3.38(s, 6H), 3.64(s, 8nH), 5.20(br, 3H), 6.73(s, 2H)

Example 10: 5kDa Double-branched Polyethylene Glycol Cyclohexane Derivative Compound

Abbreviation: 5CHTM(2EA2)

Structure:



100 mg of cis,cis-1,3,5-cyclohexane trimethanol synthesized in a manner similar to that in Example 4 was dissolved in 23 mL of dry DMSO, and then 958 mL of a tert-butanol solution (1 mol/L) of tert-butoxy potassium was added and the mixture was stirred for 1 hour at room temperature. Bromoacetate tert-butyl ester was added thereto and the mixture was stirred for 16 hours at 90°C. After the cooled solution reached room temperature it was purified with a silica gel column to obtain 22 mg of 1-0-tert-butoxycarbonyl methyl-cis,cis-1,3,5-cyclohexane trimethanol (yield: 13%).

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 0.60(m, 9H), 1.55-1.90(m, 9H), 1.48(s, 9H), 3.36(d, J=6.42Hz, 2H), 3.39(d, J=6.15Hz, 4H), 3.95(s, 2H)

< Mass Analysis (FAB-MS) >

Actual Value: $(M + H)^+$ 289

Theoretical Value: $C_{15}H_{28}O_5$ = 288

22 mg of the obtained compound above was dissolved in 200 µL of dry pyridine, in an argon atmosphere, and then 23 mg of tosyl chloride dissolved in 200 µL of dry pyridine was added. The mixture was stirred for 3 hours at 0°C and then 20 µL of water was added, after which another 100 µL was added. The reaction solution was extracted with ice-cooled chloroform and then washed sequentially with 1 mol/L ice-cooled hydrochloric acid, water, and saturated sodium bicarbonate. The solution was dried with anhydrous sodium sulfate, the solvent was removed and the product was purified with a silica gel column to obtain 22 mg of 1-0-tert-butoxycarbonyl methyl-3-O,5-O-ditosyl-1,3,5-cyclohexane trimethanol (yield: 48%).

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 1.26(m, 9H), 1.75(m, 9H), 1.47(s, 9H), 2.46(s, 6H), 3.29(d, J=6.30Hz, 2H), 3.80(m, 4H), 3.89(s, 2H), 7.36(d, J=8.10Hz, 2H), 7.76(d, J=8.40Hz, 2H)

< Mass Analysis (FAB-MS) >

Actual Value: $(M - \text{tert-butyl} + 2H)^+$ 541

Theoretical Value: $C_{29}H_{40}O_9S_2$ = 596

1.4g of mPEG(average molecular weight: 5,000, Nippon Oil and Fats, Ltd.) was dissolved in 2 mL of dry toluene, and the solution added by dripping to 26 mg of sodium hydroxide in an argon atmosphere, following which it was stirred for 30 minutes. 76 mg of 1-0-tert-butoxycarbonyl methyl-3-O,5-O-ditosyl-1,3,5-cyclohexane trimethanol dissolved in 500 µL of anhydrous toluene was added by dripping, and then the solution was stirred for one day and night at room temperature. Next, the reaction solution was added to diethyl ether by dripping and the resulting white precipitate was recovered by filtration and dried under reduced pressure. With a 120 mL DEAE Sepharose F.F. column, 1.2 g of the obtained white solid was purified in a manner similar to that in Example 2 to obtain 154 mg of the target product (yield: 11%).

< Gel Filtration HPLC Analysis >
Measurement was carried out using TSK gel $G2000SW_{XL}$ column under conditions similar to those in Example 1.

Retention time: 12.7 minutes

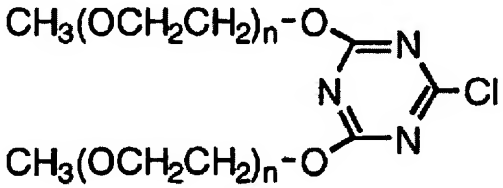
< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 3.38(s, 6H), 3.64(s, 8nH), 0.58(m, 9H), 1.72-1.93(m, 9H), 3.38(s, 6H)

Example 11: 5kDa Double-branched Polyethylene Glycol Triazine Derivative Compound

Abbreviation: PEG_2CL

Structure:



2.0 g mPEG with an average molecular weight of 5,000 (Nippon Oil and Fats, Ltd.), 444 mg of zinc oxide, and 10 mL of dry benzene were placed in a flask and heated in an oil bath to 90°~95°C, following which 4 mL of distillate was removed. The distillate was then refluxed for 5

hours and cooled to room temperature following which 36 mg of cyanyl chloride and 1 g of molecular sieve 4A were added and the mixture was dry refluxed for 3 days. The reaction solution was cooled and then centrifuged at 3,000rpm. The above purified substance was added to diethyl ether by dripping and then the resulting precipitate was recovered and dried under reduced pressure. 1 g of the obtained white solid was dissolved in a solution of 30 mg of γ-amino butyric acid in 10 mL of 0.1 mol/L borate buffer (pH 10), and was reacted for 3 days at 4°C. The solution was adjusted to a pH of between 1 and 2 by the addition of 1 mol/L hydrochloric acid and then extracted with chloroform. The chloroform was concentrated and added to diethyl ether by dripping to collect 930 mg of resulting white precipitate. The precipitate was dissolved in 930 mL of water and purified with an 80 mL DEAE Sepharose F.F. column (Amersham-Pharmacia Biotech, Inc.). The target fraction was recovered and then the pH was adjusted to between 1 and 2 with 1 mol/L of hydrochloric acid, and then an appropriate amount of chloroform was used to extract the mixture which was then concentrated under reduced pressure. The concentrated solution was dissolved in diethyl ether and the resulting precipitate was dried under reduced pressure to obtain 618 mg of target product (yield: 62%).

< Gel Filtration HPLC Analysis >

Analysis was conducted using TSK gel G-2000SW$_{XL}$ column under conditions similar to those in Example 1.

Retention time: 12.4 minutes

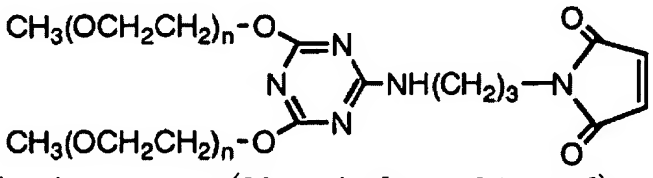
< ^{1}H-NMR Analysis (300MHz) >

δ (ppm): 2.38(t, 2H, J=6.92Hz), 1.95(m, 2H), 5.66(brt, J=6.33Hz, 1H), 4.43(brm, 2H), 3.38(s, 6H), 3.64(brs, 8nH)

Example 12: 5kDa Double-branched Polyethylene Glycol Triazine Derivative Compound

Abbreviation: PEG$_2$Mal

Structure:



2.0 mL of 1,3-diaminopropane (20 equivalents, 24 mmol) was dissolved in 600 mL of a borate buffer solution (pH 10), and 6g of the compound manufactured as described in Example 11 was stirred for one day and night. A pH of between 1 and 2 was reached by adding

2 mol/L hydrochloric acid, and the mixture was extracted with chloroform. The organic layer was washed with a saturated sodium carbonate solution and dried with anhydrous sodium sulfate. The solvent was removed under reduced pressure and methylene chloride was added to the residue, following which the solution was added to diethyl ether by dripping and the resulting white precipitate was dried under reduced pressure to obtain 4.48 mg of 2,4-bis(methoxy polyethylene glycol)-6-N-(3-aminopropyl) amino-s-triazine (yield: 75%).

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 3.64(s, 8nH), 3.38(s, 6H), 4.44(brt, 2H, J=5.0Hz), 4.49(brt, 2H, J=5.13Hz), 6.02(brt, 1H, J=5.74Hz), 2.83(t, 2H, J=6.47Hz), 1.71(m, 2H)

676 mg (8 mmol) of maleimide was dissolved in 40 mL of acetic acid ethyl, the solution was ice-cooled and 878 μL (8 mmol) of N-methyl morpholine was added, followed by the addition of 946 μL (9.6 mmol, 1.2 equivalence) of ethoxycarbonyl chloride, and then the mixture was stirred for 30 minutes. The reaction solution was filtered, following which the filtrate was washed with saturated sodium chloride and dried with anhydrous sodium sulfate. The solvent was removed under reduced pressure to obtain 654 mg of N-ethoxycarbonyl maleimide recrystallized with acetic acid methyl (diethyl) ether (yield: 55.6%).

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 6.82(s, 2H), 4.42(q, 2H, J=7.00Hz), 1.42(t, 3H, J=7.09Hz)

4.48g (0.448 mmol), 4.48g (0.448 mmol) of ice-cooled 2,4-bis(methoxy polyethylene glycol)-6-N-(3-aminopropyl) amino-s-triazine was dissolved in 45 mL of a saturated sodium bicarbonate solution, and 151.5 mg (0.90 mmol) of N-ethoxycarbonyl maleimide was added, following which the solution was stirred for 10 minutes at 0°C. 180 mL of water was added to the solution, which was then stirred for 15 minutes at room temperature. The mixture was extracted with chloroform, dried with anhydrous sodium sulfate and the solvent removed under reduced pressure. The residue was dissolved in a small amount of diethyl ether and the mixture added to diethyl ether by dripping. The resulting precipitate was filtered and the target product dried under reduced pressure to obtain 3.9g of 2,4-bis(methoxy polyethylene glycol)-6-N-(3-maleimidopropyl) amino-s-triazine (yield: 86.7%).

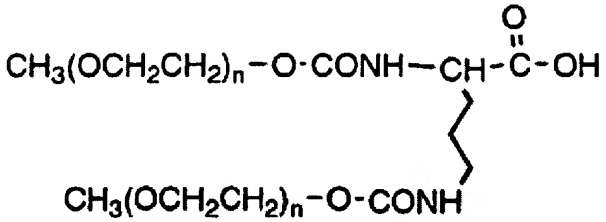
< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 3.66(s, 8nH), 3.38(s, 6H), 6.73(s, 2H), 4.46(brm, 4H), 5.76(brt, 1H, J=5.74Hz), 2.61(brt, 2H), 1.85(m, 2H)

Example 13: 5kDa Double-branched Polyethylene Glycol Ornithine Derivative Compound

Abbreviation: 5ORN(2UA)

Structure:



10g (2 mmol) of mPEG (average molecular weight: 5,000, Nippon Oil and Fats, Ltd.) was dissolved in 10 mL of methylene chloride and 2.56g (10 mmol) of DSC and 1.22g (10 mmol) of DMAP were added, following which the solution was stirred at room temperature. The insoluble material was separated by filtration and the filtrate was dissolved in 200 mol of diethyl ether to produce a precipitate. The white precipitate was filtered and dried under reduced pressure to obtain 8.6g of mPEG succinimidyl carbonate (yield: 86.0%).

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 3.67(s, 4nH), 3.38(s, 3H), 2.84(s, 4H)

Subsequently, 337.2 mg of a hydrogen chloride salt (Nakarai-Tesk, Co.) and ornithine was dissolved in 10nl of a 75 mmol/L phosphate buffer solution (pH 7.8), and the previously adjusted mPEG succinimidyl carbonate was added 1.0 g at a time and dissolved. A pH of 7.8 was maintained by adding 1 mol/L sodium hydroxide to the solution, and the solution was stirred for one day and night at room temperature. The solution was then adjusted to a pH of 3 with 1 mol/L hydrochloric acid and extracted with chloroform. The solvent was removed from the organic layer under reduced pressure, following which the residue was dissolved in a small amount of methylene chloride and the mixture was dried with diethyl ether. The white precipitate was filtered to obtain 760.2 mg of a solution wherein 1 molecule of ornithine is bonded to the mPEG (yield: 76.0%).

This product was dissolved in methylene chloride and 760.2 mg of the previously manufactured mPEG succinimidyl carbonate was added, and additionally 21.2 µL TEA was added to the mixture and stirred for one day and night at room temperature under an argon stream. The insoluble material was separated by filtration, dried under reduced pressure, and the solvent was removed, following which 50 mL of water was added and a pH of 3 maintained with 1 mol/l hydrochloric acid. The solution was extracted with chloroform and concentrated under reduced pressure. The residue was dissolved in a small amount of methylene chloride and the solution added to diethyl ether by dripping to obtain 1.21g of resulting white precipitate (yield: 79.6%). The solution was purified with a 60 mL DEAE Sepharose FF column (Amersham-Pharmacia Biotech, Inc.) to obtain 416 mg of target product (yield: 34.4%).

< Gel Filtration HPLC Analysis >

Analysis was conducted using TSK gel G-2000SW_{XL} column under conditions similar to those in Example 1.

Retention time: 12.4 minutes

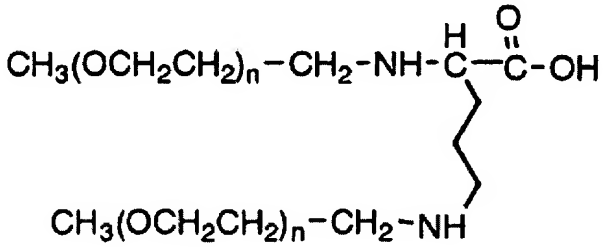
< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 5.21(br, 1H), 5.57(br, 1H), 4.1-4.4(brm, 4H), 3.64(s, 8nH), 3.38(s, 6H), 1.5-2.0(m, 6H)

Example 14: 5kDa Double-branched Polyethylene Glycol Ornithine Derivative Compound

Abbreviation: 5ORN(2RaA)

Structure:



24 mg (0.14 mmol) of an ornithine, hydrogen chloride salt (Nakarai-Tesk Co.) was suspended in 10 mL of methanol, and 1.5g (0.3 mmol) of PEG-aldehyde (Shearwater Polymers, Inc., average molecular weight: 5,000) added thereto and the mixture stirred at room temperature.

89.5 mg of sodium cyanoborohydride was added to the mixture, which was then stirred for one day and night at room temperature. Additionally, 44.8 mg (0.7 mmol) of sodium cyanoborohydride was added and the mixture was stirred. Thereafter an appropriate amount of water was added to the mixture and following removal of methanol under reduced pressure the solution was extracted with chloroform. The organic layer was dried with anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain 1.25g of raw product (yield: 83.0%).

Next, the product was purified using an SP-Sepharose FF column (120 mL, Amersham-Pharmacia Biotech, Inc.) to obtain 165 mg of target product (yield: 13.3%).

< Gel Filtration HPLC Analysis >

Analysis was conducted using TSK gel G-2000SW_{XL} column under conditions similar to those in Example 1.

Retention time: 12.4 minutes

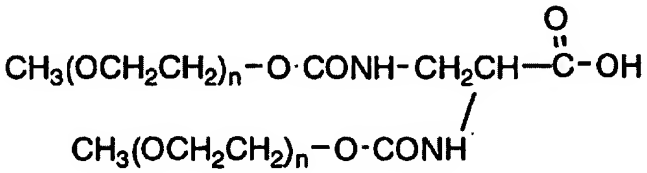
< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 1.6-2.1(m, 4H), 3.0-3.3(m, 6H), 3.38(s, 6H), 3.64(s, 8nH), 4.68(br, 1H), 4.72(br, 1H)

Example 15: 5kDa Double-branched Polyethylene Glycol Diamino Propion Derivative Compound

Abbreviation: 5DPA(2UA)

Structure:



10g (2 mmol) of mPEG (average molecular weight: 5,000, Nippon Oil and Fats, Ltd.) was dissolved in methylene chloride, and 2.56g (10 mmol) of DSC and 1.22g (10 mmol) of DMAP were added, after which the solution was stirred at room temperature. The insoluble material was separated by filtration and the filtrate was added to ethylene ether by dripping. The white precipitate was filtered and dried under reduced pressure to obtain 8.6g of mPEG succinimidyl carbonate (yield: 86.0%).

Next, 281.1 mg (2 mmol) of 2,3-diaminopropionic acid hydrochloride was dissolved in 10 mL of 75 mmol/L phosphate buffer solution (pH 7.8), and the mixture was adjusted to a pH of 8.5,.following which the previously adjusted mPEG succinimidyl carbonate was dissolved by adding 1.0 g at a time. A pH of 8.5 was maintained by adding a 1 mol/L solution of sodium hydroxide to the solution, which was then stirred for an entire day and night at room temperature. The solution was adjusted to a pH of 3 using 1 mol/L hydrochloric acid and then extracted with chloroform. The solvent was removed from the organic layer under reduced pressure to obtain 2,3-diaminopropionate in which one molecule was bound to the mPEG. This product was dissolved in methylene chloride and 730.0 mg of the previously adjusted mPEG succinimidyl carbonate was added, after which 20.4 µL of TEA was added . The solution was then stirred for one day and night at room temperature under an argon stream. The insoluble material was separated by filtration and the solvent removed under reduced pressure to obtain 1.18g of residue (yield: 89.8%). The mixture was purified with a 60 mL DEAE Sepharose FF (Amersham-Pharmacia Biotech, Inc.) *(column)* to obtain 507 mg of target product (yield: 42.9%).

< Gel Filtration HPLC Analysis >
Analysis was conducted using TSK gel G-2000SW_{XL} column under conditions similar to those in Example 1.

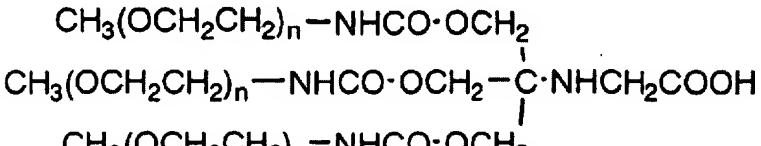
Retention time: 12.1 minutes

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >
δ (ppm): 6.00(br, 1H), 5.70(br, 1H), 4.1~4.4(brm, 4H), 3.64(s, 8nH), 3.38(s, 6H)

Example 16: 5kDa 3 Chain Branched Polyethylene Glycol Tricine Derivative Compound

Abbreviation: 5TRC(3UA)

Structure:



0.5 mg (2.8 µmol) of tricine (N-[Tris(hydroxymethyl) methyl] glycine, Nakarai-Tesk, Inc.) and 50 mg of mPEG-CO (Shearwater Polymers, Inc., average molecular weight: 5,000, structure: $CH_3(OCH_2CH_2)_n$-N=C=O) were dissolved in 0.5 mL of DMF,

1.4 μL (1.0 μmol) of TEA was added under an argon stream and then a small amount of copper chloride was added, following which the solution was stirred at room temperature. Additionally, 25 mg of PEG-NCO and 1 μL of TEA were added and the solution was stirred. Following the addition of 50 mL of 0.1 mol/L hydrochloric acid, the solution was extracted with 50 mL of chloroform. The chloroform layer was dried with anhydrous sodium sulfate and the solvent was removed under reduced pressure to obtain 15 mg of raw product (yield: 20%). Next, the product was purified with a DEAE Sepharose F.F. (Amersham-Pharmacia Biotech, Inc.) column to obtain 6.0 mg of target product (yield: 8.0%).

< Gel Filtration HPLC Analysis >

Analysis was conducted using TSK gel G-2000SW_{XL} column under conditions similar to those in Example 1.

Retention time: 11.5 minutes

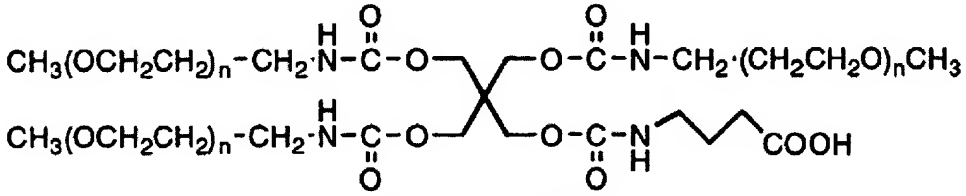
< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 3.38(s, 9H), 3.64(s, 12nH), 4.10(s, 6H), 5.43(br, 3H)

Example 17: 5kDa 3 Chain Branched Polyethylene Glycol Pentaerythritol Derivative Compound

Abbreviation: 5PET(3UA)

Structure:



136 mg of pentaerythritol and 122 mg of DMAP and 778 mg of CDI were added to 5 mL of DMF under an argon stream, and the solution was stirred for an entire day and night. 5.0g of mPEG-NH_2 (average molecular weight: 5,000, Nippon Oil and Fats, Ltd., structure: $CH_3(OCH_2CH_2)_n$-CH_2-NH_2) was dissolved in 10 mL of DMF and 1.25 mL of the previously described reaction mixture was added, following which the mixture was stirred at room temperature. The reaction solution remained in a solution of 2.6g of γ-aminobutyric acid dissolved in100 mL of a 0.1 mol/L borate buffer solution that was cooled in ice.

Upon completion of the reaction, the solution was adjusted to an acidic pH with hydrochloric acid and extracted with chloroform to obtain 4.2g of residue (yield: 84.6%). In a 1000 mL column of DEAE Sepharose (Amersham-Pharmacia Biotech, Inc.), 3.8g of residue was purified to obtain 254 mg of target product (yield: 6.7%).

< Gel Filtration HPLC Analysis >

Measurement was carried out using TSK gel G2000SW$_{XL}$ column under conditions similar to those in Example 1.

Retention time: 11.4 minutes

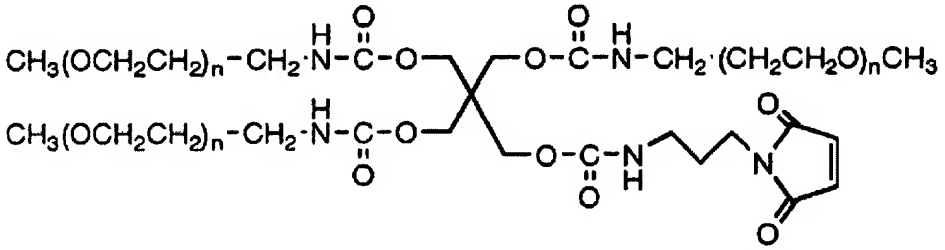
< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 5.44(brt, J=5.04Hz, 3H), 5.25(br, 1H), 4.09(brs, 8H), 3.65(s, 12nH), 3.29(s, 9H), 3.26(m, 8H), 2.37(t, J=6.8Hz, 2H), 1.80(brm, 2H), 1.77(m, 6H)

Example 18: 5kDa 3Chain Branched Polyethylene Glycol Pentaerythritol Derivative Compound

Abbreviation: 5PET(3UM)

Structure:



136 mg of pentaerythritol and 122 mg of DMAP were dissolved in 5 mL of DMF and 778 mg of CDI was added,following which the solution was stirred for one day and night under an argon stream. In 2 mL of DMF, 1.0g of mPEG-NH_2 (average molecular weight: 5,000, Structure: $CH_3(OCH_2CH_2)_n$-N-CH_2-NH_2)and 0.25 mL of the previously described reaction mixture was added thereto and the reaction proceeded at room temperature. Subsequently, a postreaction addition of 187 μL of propylene diamine was followed by an addition of diethyl ether. The white precipitate was recovered to obtain 975 mg of residue dried under reduced pressure (yield: 97.5%). The residue was purified with a 100 mL SP Sepharose F.F. column (Amersham-Pharmacia Biotech, Inc.) to obtain 110 mg of white solid (yield: 11.3%).

Then 100 mg of the white solid was dissolved in a saturated sodium bicarbonate solution and 2.3 mg of ethoxycarbonyl maleimide was added thereto and again the mixture was stirred for 10 minutes at 0°C. Following the addition of water, the mixture was stirred for 15 minutes at room temperature and extracted with chloroform. The chloroform layer was concentrated under reduced pressure then added to diethyl ether by dripping following which the white precipitate was dried under reduced pressure to obtain 35 mg of target product (yield: 35%).

< Gel Filtration HPLC Analysis >
Measurement was carried out using TSK gel G-2000SW_{XL} column under conditions similar to those in Example 1.

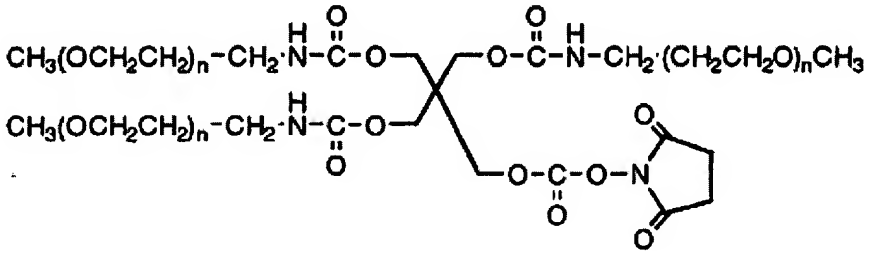
Retention time: 11.3 minutes

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >
δ (ppm): 6.73(s, 2H), 5.33(br, 3H), 4.08(brs, 8H), 3.64(s, 12nH), 3.36(s, 9H), 3.25(m, 6H), 3.11(m, 2H), 1.77(m, 8H)

Example 19: 3 Chain Branched Polyethylene Glycol Pentaerythritol Derivative Compound

Abbreviation: 5PET(3UU)

Structure:



136 mg of pentaerythritol and 122 mg of DMAP were dissolved in 5 mL of DMF, and 681 mg of CDI was added , following which the solution was stirred under an argon stream. 1.0g of mPEG-NH_2 (average molecular weight: 5,000, Nippon Oil and Fats, Ltd.,) was dissolved in 2 mL of DMF, and 286 µL of the previously described reaction mixture was added, after which the reaction proceeded at room temperature. The reaction solution was added to diethyl ether by dripping and the white precipitate was recovered and dried under reduced pressure to obtain 1g of residue (yield: 100%). Subsequently, the residue was

purified using a TSK Gel ODS-120T column (30mm X 250mm, Tosoh, Corp.) to obtain 165 mg of white powder (yield: 16.5%).

Then 80 mg of the white powder was dissolved in methylene chloride and 4.1 mg of DSC and 2.1 mg of DMAP were added, following which the solution was stirred at room temperature under an argon stream. The reaction solution was added to diethyl ether by dripping and the resulting white precipitate was dried under reduced pressure to obtain 63 mg of target product (yield: 78.8%).

< Gel Filtration HPLC Analysis >
Measurement was carried out using TSK gel G2000SW$_{XL}$ column under conditions similar to those in Example 1.

Retention time: 10.7 minutes

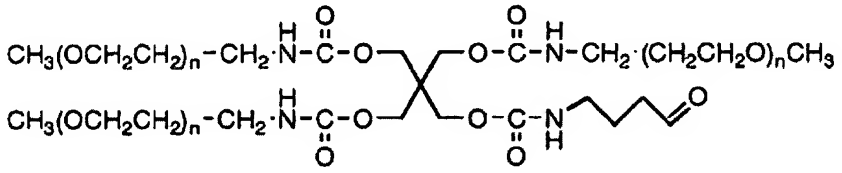
< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 5.49(br, 3H), 4.11(brs, 8H), 3.64(s, 12nH), 3.38(s, 9H), 3.25(m, 6H), 2.87(s, 4H), 1.78(m, 8H)

Example 20: 5kDa Double-branched Polyethylene Glycol Pentaerythritol Derivative Compound

Abbreviation: 5PET(3URa)

Structure:



136 mg of pentaerythritol and 122 mg of DMAP were dissolved in 5 mL of DMF, and 681 mg of CDI was added following which the solution was stirred under an argon stream. 1.0g of mPEG-NH_2 (average molecular weight: 5,000, Nippon Oil and Fats, Ltd.,) was dissolved in 2 mL of DMF, and 286 µL of the previously described reaction mixture was added,,whereupon the reaction proceeded at room temperature. The reaction solution was added

to diethyl ether by dripping and the white precipitate was recovered and dried under reduced pressure to obtain 950 mg of residue (yield: 95%). Subsequently, the residue was purified using a TSK gel ODS-120T column (30mm X 250mm, Tosoh, Corp.) to obtain 300 mg of white solid (yield: 31.6%).

The white solid was dissolved in methylene chloride and 3.5 μL of 4-aminobutyl aldehyde diethylate was added, following which the solution was stirred at room temperature. The reaction solution was added to diethyl ether by dripping and the resulting white precipitate was dried under reduced pressure to obtain 250 mg of residue (yield: 83.3%).

100 mg of residue was dissolved in methylene chloride containing 10% TFA and allowed to stand for 1 hour at 0°C, following which the white precipitate was added to diethyl ether by dripping and dried under reduced pressure to obtain 40 mg of target product (yield: 40.0%).

< Gel Filtration HPLC Analysis >

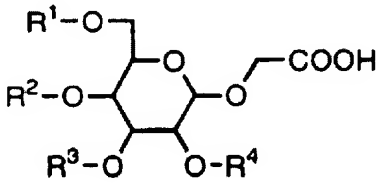
Measurement was carried out using TSK gel G-2000SW_{XL} column under conditions similar to those in Example 1.

Retention time: 10.6 minutes

Example 21: 3～4 Chain Branched Polyethylene Glycol Derivative Compound

Abbreviation: 5SUG(3UA), 5SUG(4UA)

Structure:



R^1, R^2, R^3, R^4 =
$CH_3(OCH_2CH_2)_nCH_2$-NH-CO-

and H

(In compound 5SUG(3UA), three of R^1, R^2, R^3, and R^4 are $CH_3(OCH_2CH_2)_n$-NH-CO- and the remaining two are hydrogen atoms. In compound 5SUG(4UA), R^1, R^2, R^3, and R^4 all are $CH_3(OCH_2CH_2)_n$-NH-CO-).

518g of α-D-glucose penta-acetate was dissolved in 80 mL of DMF, and 2.37g of hydrazine acetate was added, following which the solution was stirred for 1.5 hours at room temperature. The reaction solution was extracted with ethyl acetate and the ethyl acetate layer rinsed with water and saturated sodium chloride, following which the solution was dried with anhydrous sodium sulfate and concentrated under reduced pressure to obtain 4.0g of α-D-glucopyranose-2,3,4,6-tetraacetate (yield: 87%).

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

δ (ppm): 2.02(s, 3H), 2.03(s, 3H), 2.08(s, 3H), 2.10(s, 3H), 4.14(m, 1H), 4.27(m, 2H), 4.91(m, 1H), 5.09(t, 1H, J=9.7Hz), 5.47(d, 1H, J=3.7Hz), 5.55(t, 1H, J=9.8Hz)

850 mg of the compound above was dissolved in 15 mL of methylene chloride, and 4.8 mL of trichloroacetonitrile and 365 mL of DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) were added. The solution was concentrated under reduced pressure and purified using a silica gel column to obtain 635 mg of α-D-glucopyranose-2,3,4,6-tetraacetate-1-(2,2,2-trichloroethanimidate) (yield: 53%).

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

(ppm): 2.02(s, 3H), 2.04(s, 3H), 2.06(s, 3H), 2.08(s, 3H), 4.13(m, 1H), 4.21(m, 1H), 4.28(m, 1H), 5.13(m, 1H), 5.19(t, 1H, J=9.8Hz), 5.57(t, 1H, J=9.9Hz), 6.56(d, 1H, J=3.7Hz), 8.71(s, 1H)

In methylene chloride, 693 mg of the compound above and 109 µL of glycolic acid were dissolved and the solution was stirred at room temperature in an argon atmosphere. The reaction was ice-cooled and 163 µL of a mixture (2:1 ratio) of trifluoromethane sulfonic acid and dry methylene chloride was added thereto, following which the solution was stirred for one day and night. 77 µL of triethylamine was added to the mixture, which was then filtered using celite. The solution was concentrated under pressure and purified using a silica gel column to obtain 162 mg of 2,3,4,6-tetra-O-acetyl-β-D-glycopyranosyl (yield: 27%).

< ^{1}H-NMR Analysis ($CDCL_3$, 300MHz) >

(ppm): 2.01(s, 3H), 2.03(s, 3H), 2.09(s, 3H), 2.10(s, 3H), 3.70(m, 1H), 3.75(s, 3H), 4.14(m, 1H), 4.26(m, 1H), 4.29(s, 2H), 4.67(d, 1H, J=7.8Hz), 5.05(m, 1H), 5.09(t, 1H, J=10.8Hz), 5.25 (t, 1H, J=9.5Hz)

162 mg of the compound described above was dissolved in 1 mL of ethanol, and amberlyst was added.

9.4 µL of a 28% methanol solution of sodium methoxide was added and the solution was then stirred at room temperature. The reaction solution was filtered with celite and the filtrate was concentrated under reduced pressure to obtain 80 mg of [(ß-D-glucopyranosyl) oxy] acetic acid methyl ester (yield: 82%).

< ^{1}H-NMR Analysis (D2O, 300MHz) >

(ppm): 3.39(s, 2H), 3.40(m, 2H), 3.69(m, 1H), 3.75(s, 3H), 3.86(m, 1H), 4.06(m, 1H), 4.26(m, 1H), 4.44(m, 1H)

2 mg of the compound above was dissolved in 100 µL of DMF, and 7 µL of triethylamine and a small amount of CuCl were added. 240 mg of mPEG-NCO was added to the solution and the solution was stirred. The solution was added to diethyl ether by dripping and the resulting white precipitate was recovered and dried under reduced pressure. 200 mg of the obtained white solid precipitate was dissolved in a 1 mol/L solution of potassium carbonate, and the mixture was stirred for 4 hours at room temperature. Chloroform and 0.1 mL/L hydrochloric acid were added to the reaction solution and the chloroform layer was extracted, following which the solution was dried with anhydrous sodium sulfate and then dried under reduced pressure to obtain 195 mg of white solid precipitate. The precipitate was purified using a 20 mL DEAE Sepharose F.F. column (Amersham-Pharmacia Biotech, Inc.) whereby the compound indicated below was obtained.

The amount of retention time in gel filtration HPLC of a 6 mg yield (yield: 5.0%) of compound 5SUG(3UA) was 10.8 minutes. The amount of retention time in gel filtration HPLC of a 12 mg yield (yield: 7.6%) of compound 5SUG(4UA) was 10.4 minutes. Measurement was carried out using a TSK gel G2000SW$_{xl}$ column under conditions similar to those in Example 1.

< ^{1}H-NMR Analysis ($CDCl_3$, 300MHz) >

(ppm): 3.38(s, 9H or 12H), 3.64(t, 12nH or 16nH), 4.1~5.6(m, 7H)

Example 22: 10kDa Linear Chain Polyethylene Glycol Derivative Compound

Abbreviation: 10SCM

Structure:

$CH_3(OCH_2CH_2)_n$-OCH2COOH

The following method of preparation conforms to the S. Zalipsky and G. Barany method, [Journal of Bioactive and Compatible Polymers, Vol. 5, p. 227 (1990)].

10g of mPEG [monomethoxypolyethylene glycol (average molecular weight 10,000, Nippon Oil and Fats, Ltd., SUNBRIGHT VFM-3010M] was dissolved in 50 mL of dry toluene, and 1.12 g of tert-butoxy potassium added thereto, following which the mixture was distilled, with 30 mL being removed in the initial distillation. After being ice-cooled to 50°C, 1.1 mL of ethyl α-bromoacetate was added to the solution following which the solution was continuously stirred for one day and night under an argon stream. The reaction mixture was dissolved in 500 mL of diethyl ether and the resulting precipitate was recovered by filtration and dried under reduced pressure. Subsequently, 9.2 g of the obtained dry powder was dissolved in 150 mL of 1 mol/L sodium hydroxide, and the solution was stirred for 1 hour at room temperature. 60 mL of 1 mol/L hydrochloric acid was added to the solution, following which the solution was extracted with chloroform. The chloroform layer was dried with anhydrous sodium sulfate and concentrated to 10 mL under reduced pressure,and then added to 300 mL of diethyl ether by dripping, and drying the resulting precipitate under reduced pressure yielded 7.5 g of the target compound in the form of a white powder (yield: 75%).

< ^{1}H-NMR Analysis ($CDCl_3$, 300MHz) >

δ (ppm): 3.64(s, 4nH), 3.38(s, 3H), 4.15(s, 2H)

Example 23: 5kDa Double-branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5CHTO(2UU)-rhIFN-β

15 mg of the compound obtained in Example 1 (12.5 mol per mol of protein) was added to 1.2 mL of the 1.3 mg/mL solution of unmodified rhIFN-β manufactured in Example 1 in 20 mmol/l phosphate buffer (pH 7.5) containing sodium chloride, and the mixture was subjected to reaction an entire day and night at 4°C. The reaction solution was gel filtered using a 24 mL Sephacryl S300 column (Amersham-Pharmacia Biotech, Inc.). Elution was carried out using 20 mmol/L phosphate buffer containing ethylene glycol and 0.1mo/l sodium chloride. From the target product, 14.5 mL of fraction was recovered and 14.5 mL of water was added as a diluent, following which the mixture was purified with a 1.5 mL CM-Sepharose F.F. column (Amersham-Pharmacia Biotech, Inc.). The fraction obtained by gel filtration was passed through the same column followed by washing with 3 mL of the same buffer solution and then eluted with the same buffer containing 1 mol/L sodium chloride, following which the fractions were combined and then concentrated. From the fractions, 1.4 mL of a solution containing the 0.24 mg/mL target substance was recovered. (Yield: 21.5%).

<Electrophoresis>
SDS-PAGE was carried out in the presence of 2-mercaptoethanol to confirm the bands of 1 to 5 molecule-bound substances.

<Electrophoresis Conditions>
Gel: PAGEL SPG 520L (Atto Corporation)

Staining: FAST STAIN™

Molecular weight marker: Low Molecular Weight Standard (Bio-Rad Laboratories)

<Gel Filtration HPLC Analysis>

Mobile phase: 150 mmol/l sodium chloride, 20 mmol/l sodium acetate buffer (pH 4.5)

Flow rate: 0.5 mL/minute

Detection: UV 280 nm

Separation column: TSK gel G-4000SW_{XL} (7.8 X 300mm X 2 linkages) (Tosoh Corp.)

Retention time: 40.3 minutes (1 ~ 4 molecule conjugate)

Example 24: 5kDa Double-branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5CHTC(2AA)-rhIFN-β

100 mg (0.01 mmol) of the compound in Example 2 was dissolved in 1.0 mL of methylene chloride, and 5.7 mg (0.05 mmol) of NHS and 10.3 mg (0.05 mmol) of DCC were added. The solution was then stirred for 30 minutes under an argon stream at 0°C., followed by stirring for 3 hours at room temperature, and then the reaction solution was added to diethyl ether by dripping. From the compound in Example 2 wherein the white precipitate was dried under reduced pressure, 65.0 mg of NHS ester was obtained (yield: 65.0%).

17 mg of the NHS ester described above (25 mol per mol of protein) was added to 1.28 mL of a (1.067 mg/mL) solution of the unmodified rhIFN-β obtained in Example 1 adjusted with a 20 mmol/L phosphate buffer (pH 7.5) containing sodium chloride,

and the mixture was subjected to reaction an entire day and night at 4°C. The reaction solution was gel filtered using a 24 mL Sephacryl S300 column (Amersham-Pharmacia Biotech, Inc.). Elution was carried out using 20 mmol/L phosphate buffer containing ethylene glycol and 0.1 mol/L sodium chloride. 24 mL of fraction was recovered from the target product, and 24 mL of water was added as a diluent, following which the mixture was purified with a 1.5 mL CM-Sepharose F.F. column (Amersham-Pharmacia Biotech, Inc.). The fraction obtained by gel filtration was passed through the same column followed by washing with 3 mL of the same buffer solution and then eluted with the same buffer containing 1 mol/L sodium chloride, following which the fractions were combined and then concentrated. From the fractions, 1.4 mL of a solution containing the 0.49 mg/mL target substance was recovered. (Yield: 44.5%).

<Electrophoresis>
SDS-PAGE was carried out using a method similar to that in Example 23 to confirm the bands of 1 to 4 molecule-bound substances.

< Gel Filtration HPLC Analysis >
Analysis was conducted using 2 TSK gel G4000SW$_{XL}$ columns under conditions similar to those in Example 23.

Retention time: 43.9 minutes (1 molecule conjugate)
41.0 minutes (2 molecule-bound substance)

Example 25: 5kDa Double-branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5CHTO(2EA)-rhIFN-β

20 mg of the compound in Example 32 was dissolved in methylene chloride, and 1.15 mg of NHS and 2.06 of DCC were added thereto, following which the solution was ice-cooled for 30 minutes and subsequently stirred for 2 hours at room temperature under an argon stream. Insoluble material was filtered and the filtrated added to diethyl ether by dripping and precipitated. 14.5 mg of NHS ester was obtained from the compound in Example 3 wherein the white precipitate was dried under reduced pressure, (yield: 72.5%).

9.1 mg of the NHS ester described above (25 mol per mol of protein) was added to 0.78 mL of a (0.937 mg/mL) solution of unmodified rhIFN-β obtained in Reference Example 1 adjusted with a 20 mmol/L phosphate buffer (pH 7.5) containing ethylene glycol and sodium chloride and the mixture was subjected to reaction an entire day and night at

4°C. The reaction solution was gel filtered using a 24 mL Sephacryl S300 column (Amersham-Pharmacia Biotech, Inc.). Elution was carried out using 20 mmol/L phosphate buffer containing ethylene glycol and 0.1mo/l sodium chloride. From the target product, 8.5 mL of fraction was recovered and 8.5 mL of water was added as a diluent, following which the mixture was purified with a 1.5 mL CM-Sepharose F.F. column (Amersham-Pharmacia Biotech, Inc.). The fraction obtained by gel filtration was passed through the same column followed by washing with 3 mL of the same buffer solution and then eluted with the same buffer containing 1 mol/L sodium chloride, following which the fractions were combined and then concentrated. From the fractions, 0.5 mL of a solution containing the 0.067 mg/mL target substance was recovered. (Yield: 44.5%).

<Electrophoresis>
SDS-PAGE was carried out using a method similar to that in Example 23 to confirm the bands of 1 to 3 molecule-bound substances.

< Gel Filtration HPLC Analysis >
Analysis was conducted using 2 TSK gel G4000SW_{XL} columns under conditions similar to those in Example 23.

Retention time: 35.9 minutes (1 ~ 3 molecule-bound substance)

Example 26: 5kDa Double-branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5CHTM(2EA)-rhIFN-β

487 (48.7 μmol) of the compound in Example 4 was dissolved in methylene chloride, and 16.8 mg (146 μmol) of NHS and 30.1 (145.9 μmol) were added , following which the solution was ice-cooled for 30 minutes then stirred for 2 hours at room temperature under an argon stream. Insoluble material was filtered and the filtrate was added to diethyl ether by dripping and then precipitated. 260.0 mg of NHS ester was obtained from the compound in Example 3 by drying the white precipitate under reduced pressure (yield: 53.4%).

14.6 mg of the NHS ester described above (20 mol per mol of protein) was added to 1.2 mL of a (1.22 mg/mL) solution of the unmodified rhIFN-β obtained in Example 1 adjusted with a 20 mmol/L phosphate buffer (pH 7.8) containing ethylene glycol and sodium chloride, and the mixture was subjected to reaction for one day and night at 4°C.

Using a gel filter Sephacryl-G25 column (NAP-10Amersham-Pharmacia Biotech, Inc.), the reaction solution was subjected to buffer exchange with 20 mmol phosphate buffer (pH 6.0) containing ethylene glycol. The fraction obtained by gel filtration was passed through a 1.5 mL CM Sepharose F.F. column (Amersham-Pharmacia Biotech, Inc.) and after washing with 3 mL of 20 mmol/L phosphate buffer (pH 6.0) was eluted with the buffer containing 0.2 ~ 1.0 mol/L sodium chloride, and the target fractions were combined and concentrated to collect 3.75 mL of fraction containing 0.194 mg/mL of the target substance. (Yield: 49.7%).

<Electrophoresis>

Analysis was conducted in a manner similar to that in Example 23 to confirm the polyethylene glycol 1- and 2-molecule-bound conjugate.

< Gel Filtration HPLC Analysis >

Analysis was conducted using 2 TSK gel G-4000SW$_{XL}$ columns under conditions similar to those in Example 23.

Retention time: 42.9 minutes (1 molecule conjugate)
40.2 minutes (2 molecule-bound substance)

Example 27: 5kDa Double-branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5ORN(2UA)-rhIFN-β

100 mg of the compound in Example 13 was dissolved in 1 mL of methylene chloride, and 3.5 mg (30 μmol) of NHS and 6.2 mg (30 μmol) of DCC were added , following which the solution was ice-cooled for 30 minutes and subsequently stirred for 2 hours at room temperature under an argon stream. Insoluble material was filtered and the filtrate added to diethyl ether by dripping and precipitated. The precipitate was dried under reduced pressure and 65 mg of NHS ester was obtained (yield: 65%) from the compound in Example 13.

12 mg of the activated modified agent 1 described above (20 mol per mol of protein) was added to 1.2 mL of a (1.0 mg/mL) solution of rhIFN-β obtained in Example 1 adjusted with a 20 mmol/L phosphate buffer containing ethylene glycol and 1 mol/L sodium chloride, and the mixture was subjected to reaction for one day and night at 4°C.

The reaction solution was purified in the same manner as that in Example 23 with a 1.5 mL CM-Sepharose FF column (Amersham-Pharmacia Biotech, Inc.). to obtain 4.4 mL of 0.113 mg/mL target substance (yield: 41.4%).

<Electrophoresis>

Analysis was conducted in a manner similar to that in Example 23 to confirm the bands of 1～4 molecule-bound substances.

< Gel Filtration HPLC Analysis >

Analysis was conducted using 2 TSK gel G-4000SW$_{XL}$ columns under conditions similar to those in Example 23.

Retention time: 43.1 minutes (1 molecule conjugate)

40.0 minutes (2 molecule-bound substance)
38.5 minutes (3 molecule-bound substance)

Example 28: 5kDa Double-branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5ORN(2RaA)-rhIFN-β

100 mg (10 μmol) of the compound in Example 14 was dissolved in 1 mL of methylene chloride and 5.7 mg (50 μmol) of NHS and 10.3 mg (50 μmol)of DCC were added, following which the solution was ice-cooled for 30 minutes and subsequently stirred for 2 hours at room temperature under an argon stream. Insoluble material was removed by filtering and the filtrate was added to diethyl ether by dripping. The precipitate was dried under reduced pressure, and 58 mg of NHS ester was obtained from the compound in Example 14 (yield: 58%).

14.6 mg of the NHS ester described above (20 mol per mol of protein) was added to 1.2 mL of a (1.2 mg/mL) solution of rhIFN-β adjusted with a 20 mmol/L phosphate buffer containing ethylene glycol and 1 mol/L sodium chloride and the mixture was subjected to reaction.

The reaction solution was purified with a 1.5 mL CM-Sepharose FF column (Amersham-Pharmacia Biotech, Inc.) to obtain 3.5 mL of 0.12 mg/mL target substance (yield: 35.0%).

<Electrophoresis>

SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1 to 3 molecule conjugates.

< Gel Filtration HPLC Analysis >

Analysis was conducted using 2 TSK gel G-4000SW_{XL} columns under conditions similar to those in Example 23.

Retention time: 43.5 minutes (1 molecule conjugate)
41.1 minutes (2 molecule conjugate)

Example 29: 5kDa Double-branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5DPA(2UA)-rhIFN-β

100 mg (10 μmol) of the compound in Example 15 was dissolved in 1 mL of methylene chloride and 5.75 mg (50 μmol) of NHS and 10.3 mg (50 μL) of DCC were added, following which the solution was ice-cooled for 30 minutes and subsequently stirred for 2 hours at room temperature under an argon stream. Insoluble material was filtered and the filtrate added to diethyl ether by dripping. The precipitate was dried under reduced pressure, and 71 mg of NHS ester was obtained from the compound in Example 15 (yield: 71).

13.2 mg of the NHS ester described above (20 mol per mol of protein) was added to 1.2 mL of a (1.1 mg/mL) solution of rhIFN-β adjusted with a 20 mmol/L phosphate buffer containing ethylene glycol and 1 mol/L sodium chloride and the mixture was subjected to reaction. The reaction solution was purified with a 1.5 mL CM-Sepharose FF column (Amersham-Pharmacia Biotech, Inc.) to obtain 3.5 mL of 0.12 mg/mL target substance (yield: 31.8%).

<Electrophoresis>

SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1 to 3 molecule conjugates.

< Gel Filtration HPLC Analysis >

Analysis was conducted using 2 TSK gel G-4000SW_{XL} columns under conditions similar to those in Example 23.

Retention time: 43.0 minutes (1 molecule conjugate)
48.2 minutes (2 molecule conjugate)

Example 30: 6kDa Double-branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: PEG2Mal-rhIFN-β

2.7 mg (average molecular weight: 12,000) of the compound obtained in Example 12 (3 mol per mol of protein) was added to 0.62 mL of a 2.44 mg/mL solution of rhIFN-β adjusted with a 20 mmol/L phosphate buffer containing sodium chloride, carbamide, and mannitol, and the mixture was subjected to reaction. A 0.38 mL quantity of reaction solution was purified by gel filtration with a Sephacryl S-300 column (column size: 20 mL, Amersham-Pharmacia Biotech, Inc.) whereby 1.5 mL of fraction containing 0.17 mg/mL of the target substance was recovered (yield: 16.9%).

<Electrophoresis>

SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1 molecule conjugates.

< Gel Filtration HPLC Analysis >

Measurement was carried out using 2 TSK gel G-4000SW_{XL} columns under conditions similar to those in Example 23.

Retention time: 44.4 minutes (1 molecule conjugate)

Example 31: 20kDa Linear Chain Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 20Mal-rhIFN-β

2.1 mg (3 mol per mol of protein) of mPEG-maleimide (average molecular weight: 20,000, Shearwater Polymers, Inc.) was added to 0.42 mL of a 1.72 mg/mL solution of rhIFN-β adjusted with a 20 mmol/L phosphate buffer containing sodium chloride, carbamide, and mannitol, and the mixture was subjected to reaction. A 0.38 mL quantity of reaction solution was purified by gel filtration with a Sephacryl S-400 column (column size: 20 mL, Amersham-Pharmacia Biotech, Inc.) whereby 1.5 mL of fraction containing 0.10 mg/mL of the target substance was recovered (yield: 22.7%).

<Electrophoresis>
SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1 molecule conjugates.

< Gel Filtration HPLC Analysis >
Measurement was carried out using 2 TSK gel G-4000SW_{XL} columns under conditions similar to those in Example 23.

Retention time: 40.0 minutes

Example 32: Preparation of 5kDa Linear Chain Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5Mal-rhIFN-β

0.55 mg (3 mol per mol of protein) of mPEG-maleimide (average molecular weight: 5,000, Shearwater Polymers, Inc.) was added to 0.42 mL of a 1.7 mg/mL solution of rhIFN-β adjusted with a 20 mmol/L phosphate buffer containing sodium chloride, carbamide, and mannitol, and the mixture was subjected to reaction. A 0.38 mL quantity of reaction solution was purified by gel filtration with a Sephacryl S-200 column (column size: 20 mL, Amersham-Pharmacia Biotech, Inc.) thereby collecting 4.5 mL of target fraction from which associated substances and non responsive rhIFN-β were removed. The fraction was purified with a CM-Sepharose FF column (column size: 1 mL, Amersham-Pharmacia Biotech, Inc) whereby 1 mL of fraction containing 0.082 mg/mL of the target substance was recovered (yield: 11.3%).

<Electrophoresis>

SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the band of 1 molecule conjugates.

< Gel Filtration HPLC Analysis >

Measurement was carried out using 2 TSK gel G-4000SW_{XL} columns under conditions similar to those in Example 23.

Retention time: 46.2 minutes

Example 33: 5kDa 3-Chain Branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5TRC(3UA)-rhIFN-β

50 μL (0.66 μmol) of a 1/5 mg/mL NHS solution adjusted with sodium chloride and 100 μL (0.66 μmol) of a 1.4 mg/mL DCC solution were added to 5 mg (0.33 μmol) of the compound in Example 16, and ice-cooled for 30 minutes then stirred for 2 hours at room temperature under an argon stream. The resulting precipitate from the diethyl ether addition was dried under reduced pressure to obtain 3.5 mg of NHS ester (yield: 70%).

33.4 mg of the NHS ester described above (34 mol per mol of protein) was added to 150 μL (0.9 mg/mL) of the rhIFN-β solution obtained in Example 1 adjusted with a 20 mmol/L phosphate buffer containing ethylene glycol and sodium chloride, and the mixture was subjected to reaction while allowed to stand for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L phosphate buffer (pH 6.0) containing ethylene glycol by means of a gel filter Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.), then purified with a CM Sepharose F.F. 0.5 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.40 mL of 0.091 mg/mL target substance (yield: 27%).

<Electrophoresis>

SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugates.

< Gel Filtration HPLC Analysis >

Analysis was carried out using 2 TSK gel G4000SW_{XL} columns under conditions similar to those in Example 23.

Retention time: 42.0 minutes (1 molecule conjugate)
44.1 minutes (2-molecule conjugate)

Example 34: 5kDa 3-Chain Branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5SKA(3UA)-rhIFN-β

16 mg (1.1 μmol) of the 5SKA(3UA) compound in Example 7 was dissolved in 100 μL of sodium chloride, and 272 μg of DCC and 152 μg of NHS were added, following which the mixture was ice-cooled for 1 hour, and stirred for 1 hour at room temperature. The white precipitate formed from the mixture being added to diethyl ether by dripping was dried under reduced pressure to obtain 14.5 mg of the NHS ester compound described above (yield: 91%).

8.6 mg of the NHS ester (34 mol per mol of protein) obtained as described above was added to a 100 μL (1.2 mg/mL) solution of rhIFN-β obtained in Example 1 adjusted with a 20 mmol/L phosphate buffer containing ethylene glycol and sodium chloride, and the mixture was subjected to reaction while allowed to stand for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L phosphate buffer (pH 6.0) containing ethylene glycol by means of a gel filter Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.), then purified with a CM Sepharose F.F. 0.6 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 80 μL of 47 μg/mL target substance (yield: 3.3%).

<Electrophoresis>
SDS-PAGE was carried out in the presence of 2-mercaptethanol in a manner similar to that in Example 23 to confirm the band of 1 molecule conjugates.

< Gel Filtration HPLC Analysis >
Analysis was carried out using 2 TSK gel G4000SW$_{XL}$ columns under conditions similar to those in Example 23.

Retention time: 41.7 minutes (1 molecule conjugate)

Example 35: 5kDa 3-Chain Branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5PET(3UU)-rhIFN-β

4.5 mg of the 5PET(3UU) compound (10 mol per mol of protein) obtained in Example 19 was added to a 0.5 mL (1.2 mg/mL) solution of the rhIFN-β obtained in Example 1 adjusted with a 20 mmol/L phosphate buffer (pH 7.8) containing ethylene glycol and sodium chloride, following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L phosphate buffer (pH 6) containing ethylene glycol by means of a Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.).

The reaction solution was purified with a CM Sepharose F.F. 0.8 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.36 mL of 0.67 mg/mL target substance (yield: 40%).

<Electrophoresis>

SDS-PAGE was carried out in the presence of 2-mercaptethanol in a manner similar to that in Example 23 to confirm the band of 1～3 molecule conjugates.

< Gel Filtration HPLC Analysis >

Analysis was carried out using 2 TSK gel G4000SW$_{XL}$ columns under conditions similar to those in Example 23.

Retention time: 41.1 minutes (1 molecule conjugate)
38.2 minutes (2 molecule conjugate)

Example 36: Preparation of 5kDa 3-Chain Branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5PET(3UA)-rhIFN-β

254 mg (0.02 mmol) of the 5PET(3UA) compound in Example 17 was dissolved in 2.0 mL of sodium chloride, and 5.9 mg (0.05 mmol) and 10.5 mg (0.05 mmol) of DCC were added, following which the mixture was stirred for 2 hours at room temperature in a stream of argon for 1 hour at 0°C. The reaction solution was added to diethyl ether by dripping and the resulting precipitate was dried under reduced pressure to obtain 132.8 mg of the NHS ester compound in Example 17 (yield: 52.3%).

13 mg of the NHS ester compound of Example 17 (15 mol per mol of protein) was added to 1.0 mL (1.16 mg/mL) solution of the rhIFN-β obtained in Reference Example 1 prepared with a 20 mmol/L phosphate buffer (pH 7.8) containing ethylene glycol and sodium chloride, following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L phosphate buffer (pH 6) containing ethylene glycol by means of a Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.). The reaction solution was purified with a CM Sepharose F.F. 1.4 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 1.0 mL of 0.14 mg/mL target substance (yield: 12%).

<Electrophoresis>

SDS-PAGE was carried out in the presence of 2-mercapthanol in a manner similar to

that in Example 23 to confirm the bands of 1～3 molecule conjugates.

< Gel Filtration HPLC Analysis >
Analysis was carried out using 2 TSK gel G4000SW_{XL} columns under conditions similar to those in Example 23.

Retention time: 43.8 minutes (1 molecule conjugate)
41.2 minutes (2 molecule conjugate)

Example 37: Preparation of Recombinant Human Interferon-β Modified with 5KDa Double-Chain Branched Polyethylene Glycol (Conventional Reagent)

Abbreviation: PEG_2Lys-rhIFN-β

8.3 mg (12.5 mol per mol of protein) of PEG_2Lys (average molecular weight: 10,000, Shearwater Polymers, Inc.) was added to a 1.3 mL (0.97 mg/mL) solution of the rhIFN-β obtained in Reference Example 1 adjusted with a 20 mmol/L phosphate buffer (pH 7.8) containing ethylene glycol and sodium chloride, following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L sodium acetic acid (pH 6) containing ethylene glycol by means of a Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.). The mixture was purified with an SP-Sepharose F.F. 1.4 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 2.7 mL of a solution containing 0.36 mg/mL target substance (yield: 76.7%).

<Electrophoresis>
SDS-PAGE was carried out in the presence of 2-mercaptoethanol in a manner similar to Example 23 to confirm the bands of 1 to 3 molecule conjugates.

<Gel Filtration HPLC Analysis>
Analysis was conducted using two TSK gel G4000SW_{XL} columns in a manner similar to that in Example 23.

Retention time: 45.3 minutes (1 molecule conjugate)
41.5 minutes (2-molecule conjugate)

Example 38: Preparation of Recombinant Human Interferon-β Modified with 20KDa Linear Chain Branched Polyethylene Glycol

Abbreviation: 20SPA-rhIFN-β

7.9 mg (6 mol per mol of protein) of an mPEG – Propionic acid NHS ester (average molecular weight: 20,000, Shearwater Polymers, Inc.) was added to 1.3 mL of 1.0 mg/mL rhIFN-β adjusted with a 20 mmol/L phosphate buffer (pH 7.5), following which the mixture was subjected to reaction while allowed to stand for an entire day and night at 4°C. The mixture was subjected to buffer exchange with a 20 mmol/L sodium acetic acid (pH 6.0) using a Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.) to obtain 2.1 mL of 0.52 mg/mL reaction solution. Next, 1.9 mL of solution was purified with an SP-Sepharose F.F. 1.9 mL column (Amersham-Pharmacia Biotech, Inc.) whereby 4.9 mL of fraction containing 0.18 mg/mL target substance was recovered (yield: 66.5%).

<Electrophoresis>

SDS-PAGE was carried out in a manner similar to Example 23 to confirm the bands of 1 to 3 molecule conjugates.

<Gel Filtration HPLC Analysis>

Analysis was conducted using two TSK gel G-4000SW_{XL} columns in a manner similar to that in Example 23.

Retention time: 40.1 minutes (1 molecule conjugate)
35.6 minutes (2 molecule conjugate)
33.2 minutes (3 molecule conjugate)

Example 39: Preparation of Recombinant Human Interferon-β Modified with 5KDa Linear Chain Branched Polyethylene Glycol

Abbreviation: 5SPA-rhIFN-β

1.6 mg (6 mol per mol of protein) of an mPEG – Propionic acid NHS ester (average molecular weight: 5,000, Shearwater Polymers, Inc.) was added to 1.3 mL of 0.8 mg/mL rhIFN-β adjusted with a 20 mmol/L phosphate buffer (pH 7.5), following which the mixture was subjected to reaction while allowed to stand for an entire day and night at 4°C. The mixture was subjected to buffer exchange with a 20 mmol/L phosphate buffer (pH 6.0) using a Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.) to obtain 2.1 mL of 0.40 mg/mL reaction solution. Next, 1.9 mL of solution was purified with an SP-Sepharose F.F. 1.5 mL column (Amersham-Pharmacia Biotech, Inc.) whereby 4.2 mL of fraction containing 0.15 mg/mL target substance was recovered (yield: 60.1%).

<Electrophoresis>

SDS-PAGE was carried out in a manner similar to Example 23 to confirm the bands of 1 to 3 molecule conjugates.

<Gel Filtration HPLC Analysis>
Analysis was conducted using two TSK gel G-4000SW$_{XL}$ columns in a manner similar to that in Example 23.

Retention time: 46.2 minutes (1 molecule conjugate)
43.6 minutes (2 molecule conjugate)
42.0 minutes (3 molecule conjugate)

Example 40: Preparation of Recombinant Human Interferon-β Modified with 10KDa Linear Chain Branched Polyethylene Glycol

Abbreviation: 10SCM-rhIFN-β

1.0g (0.1 mmol) of the compound in Example 22 was dissolved in methylene chloride and 21.8 mg (0.19 mmol) of NHS and 39.0 mg (0.19 mmol) of DCC were added following which the mixture was ice-cooled for 30 minutes and subsequently stirred for 2 hours at room temperature under an argon stream. Insoluble material was filtered and the filtrate added to diethyl ether by dripping and then precipitated. The precipitate was dried under reduced pressure to obtain 506.8g of the NHS ester, the compound in Example 22 (yield: 50.7%).

16.2 mg of the NHS ester described above was added to 3.0 mL solution of (0.81 mg/mL) the rhIFN-β obtained in Reference Example 1 adjusted with a 20 mmol/L phosphate buffer (pH 7.8) containing ethylene glycol and sodium chloride, following which the mixture was subjected to reaction for an entire day and night at 4°C. Next, the mixture was dechlorinated using a Sephadex-G25 gel filtration column (Amersham-Pharmacia Biotech, Inc.). A 4.5 mL quantity of fraction obtained by gel filtration was purified by means of a CM Sepharose F.F. 2.0 mL column (Amersham-Pharmacia Biotech, Inc.) whereby 4.0 mL of fraction containing 0.22 mg/mL target fraction was recovered (yield: 36.2%).

<Electrophoresis>
Analysis was conducted in a manner similar to that in Example 23 to confirm the polyethylene glycol 1～3 molecule-bound conjugate.

<Gel Filtration HPLC Analysis>
Analysis was conducted using two TSK gel G-4000SW$_{XL}$ columns in a manner similar to that in Example 23.

Retention time: 44.2 minutes (1 molecule conjugate)
41.0 minutes (2 molecule conjugate)

Example 41: Preparation of Natural Human Interferon-β Modified with 5KDa Double-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTM(2EA)- natural rhIFN-β

10 μg of natural hIFN-β (Strathmann Biotech BMBH was dissolved in 200 μL of isotonic phosphate buffer, and 1.5 mg of 5CHTM(2EA) NHS ester (300 mol per mol protein) obtained in Example 26 was added, following which the mixture was subjected to reaction for an entire day and night at 20°C. The reaction solution was subjected to buffer exchange with 20 mmol/l phosphate buffer containing ethylene glycol by means of a gel filtration Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.), then purified with a CM Sepharose F.F. 0.5 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.19 mL of 0.021 mg/mL target substance (yield: 39.9%).

Example 42: Preparation of 5kDa 2-Chain Branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5CHTM(2EA)-^{17}Ser rhIFN-β

1.3 mg of (25 mol per mole of protein) NHS ester compound (5CHTM(2EA) of Example 4 obtained in a manner similar to that of Example 26 was added to 0.1 mL (1.0 mg/mL) of ^{17}Ser rhIFN-β solution adjusted with a 20 mmol/L phosphate buffer (pH 7.6) containing ethylene glycol and sodium chloride, following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L phosphate buffer (pH 6.0) containing ethylene glycol by means of a gel filtration Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.). The fraction obtained from gel filtration was purified with a CM Sepharose F.F. 0.25 mL column (Amersham-Pharmacia Biotech, Inc.), and 0.75 mL of fraction containing 39 μg of target substance was recovered (yield: 39%).

<Electrophoresis>

Analysis was conducted in a manner similar to that in Example 23 to confirm the polyethylene glycol 1～3 molecule-bound conjugate.

<Gel Filtration HPLC Analysis>

Analysis was conducted using two TSK gel G-4000SW$_{XL}$ columns in a manner similar to that in Example 23.

Retention time: 41.3 minutes (1 ～ 3 molecule conjugate)

Example 43: Preparation of 5kDa 3-Chain Branched Polyethylene Glycol Modified Recombinant Human Interferon-β Preparation

Abbreviation: 5PET(3UA)-^{17}Ser rhIFN-β

1.6 mg of (20 mol per mole of protein) the NHS ester compound (5PET(3UA)) of Example 17 obtained in a manner similar to that of Example 36 was added to 0.05 mL (2.1 mg/mL) of ^{17}Ser rhIFN-β solution (Chiron Corp.) adjusted with a 20 mmol/L phosphate buffer (pH 7.5) containing ethylene glycol and sodium chloride, following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L phosphate buffer (pH 6) containing ethylene glycol using a Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.). The fraction obtained from gel filtration was purified with a CM Sepharose F.F. 0.25 mL column (Amersham-Pharmacia Biotech, Inc.), and 0.30 mL of fraction containing 27.8 μg/mL of target substance was recovered (yield: 7.9%).

<Electrophoresis>

SDS-PAGE was carried out in the presence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm bands of a polyethylene glycol 1～3 molecule-bound conjugate.

Example 44: Preparation of 5kDa 2-Chain Branched Polyethylene Glycol Modified Recombinant Human Interferon-α Preparation

Abbreviation: 5CHTC(2AA)-rhIFN-α

1.5 mg of (30 mol per mole of protein) NHS ester (5CHTC(2AA) obtained in Example 24 was added to 0.1 mL of 1.0 mg/mL rhIFN-α [Immuno-Biological Laboratories, Inc. (IBL)] prepared with an isotonic phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. A 80 μL quantity of reaction solution was subjected to buffer exchange with a 20 mmol/L phosphate buffer (pH 4.5) by means of a Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.) and 0.8 mL of solution recovered. This solution was purified by means of a SP-Sepharose F.F. 1.0 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.5 mL of reaction solution containing 80 μg/mL of target substance.(yield: 40.0%).

<Electrophoresis>

SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule-bound substance.

<Gel Filtration HPLC Analysis>

Analysis was conducted using a TSK gel G-4000SW$_{XL}$ column in under conditions similar to those described in Example 23.

Retention time: 43.1 minutes (1 molecule conjugate)
40.5 minutes (2 molecule conjugate)

Example 45: Preparation of 5kDa 2-Chain Branched Polyethylene Glycol Modified Recombinant Human Interferon-α Preparation

Abbreviation: 5CHTM(2EA)-rhIFN-α

1.5 mg (30 mol per mole of protein) of the NHS ester (5CHTM(2EA) obtained in Example 26 was added to 0.1 mL of 0.95 mg/mL rhIFN-α [Immuno-Biological Laboratories, Inc. (IBL)] prepared with an isotonic phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. A 0.1 mL quantity of reaction solution was subjected to buffer exchange with a 20 mmol/L phosphate buffer (pH 4.5) by means of a Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.) and 0.8 mL of solution was recovered. This solution was purified by means of a SP-Sepharose F.F. 1.0 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.6 mL of reaction solution containing 50 μg/mL of target substance.(yield: 31.6%).

<Electrophoresis>

SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1- and 2-molecule-bound substance.

<Gel Filtration HPLC Analysis>

Analysis was conducted using a TSK gel G-4000SW$_{XL}$ column under conditions similar to those described in Example 23.

Retention time: 42.9 minutes (1 molecule conjugate)
41.2 minutes (2 molecule conjugate)

Example 46: Preparation of 5kDa 3-Chain Branched Polyethylene Glycol Modified Recombinant Human Interferon-α Preparation

Abbreviation: 5PET(3UA)-rhIFN-α

1.6 mg (20 mol

per mole of protein) of the NHS ester compound (5PET(3UA)) obtained in Example 36 was added to 0.1 mL of a 1.0 mg/mL rhIFN-α solution [Immuno-Biological Laboratories, Inc. (IBL)] prepared with an isotonic phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L phosphate buffer (pH 4.5) by means of a Sephacryl-G25 column (Amersham-Pharmacia Biotech, Inc.). This solution was purified by means of a SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 65 μL of reaction solution containing 0.53 mg/mL of target substance.(yield: 34%).

<Electrophoresis>
SDS-PAGE was carried out in the presence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule-bound substance.

<Gel Filtration HPLC Analysis>

Analysis was conducted using 2 TSK gel G4000SW$_{XL}$ column2 in a manner similar to that in Example 23.

Retention time: 42.6 minutes (1-molecule conjugate)
40.3 minutes (2-molecule conjugate)

Example 47: Preparation of 5kDa 2-Chain Branched Polyethylene Glycol Modified Recombinant Human Interferon-γ Preparation

Abbreviation: 5CHTC(2AA)-rhIFN-γ

1.0 mg (200 mol per mole of protein) of the NHS ester (5CTC(2AA)) obtained in Example 24 was added to 0.1 mL (0.10 mg/mL) of the rhIFN- γ solution obtained in Reference Example 2 prepared with a 20m mL phosphate buffer (pH 7.8) containing ethylene glycol and sodium chloride, following which the mixture was subjected to reaction for an entire day and night at 4°C.

<Electrophoresis>
SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

Example 48: Preparation of 5kDa 2-Chain Branched Polyethylene Glycol Modified Recombinant Human Interferon-γ Preparation

Abbreviation: 5CHTM(2EA)-rhIFN-γ

1.0 mg (200 mol per mole of protein) of the NHS ester (5CHTM(2EA) obtained in Example 26 to the 0.8 mL of (0.8 mg/mL) rhIFN-γ obtained in Reference Example 2 prepared with a

20m mL phosphate buffer (pH 7.8) containing ethylene glycol and sodium chloride, following which the mixture was subjected to reaction for an entire day and night at 4°C.

<Electrophoresis>

SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

Example 49: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa Three-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTO(2UU)-rhG-CSF Derivative

1.7 mg of the compound (10 mol per mole of protein) in Example 1 was added to 100 µL of 32 mg/mL rhG-CSF derivative obtained in Reference Example 3 prepared with 50 mmol/L of phosphate buffer, following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was diluted with 20 mmol/L acetic acid (pH 4.5), following which 900 µL of the solution was passed through a Sephadex G-25 column equilibrated with the same buffer solution and 1.3 mL of the mixture was recovered. The mixture was purified by means of an SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 360 µL of reaction solution containing 402 µg/mL of target substance.(yield: 50.3%).

<Electrophoresis>

SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～4 molecule conjugate.

<Gel Filtration HPLC Analysis>

Analysis was conducted using 2 TSK gel G4000SW$_{XL}$ columns in a manner similar to that in Example 23.

Retention time: 42.8 minutes (1-molecule conjugate)
41.3 minutes (2-molecule conjugate)

Example 50: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa Three-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTO(2AA)-rhG-CSF Derivative

5.1 mg (25 mol per mol of protein) of the NHS ester (5CHTC(2AA)) obtained in Example 24 was added to 100 µL of 32 mg/mL rhG-CSF derivative obtained in Reference Example 3 adjusted to 3.9 mg/mL with a 50 mmol/l phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was diluted and 900 µL of the solution was subjected to buffer exchange with a 20 mmol/L acetic acid buffer, whereby 1.3 mL of solution was recovered. This was purified by means of an SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 500 µL of reaction solution containing 179 µg/mL of target substance.(yield: 25.8%).

<Electrophoresis>

SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

<Gel Filtration HPLC Analysis>

Analysis was conducted using 2 TSK gel G4000SW$_{XL}$ columns in a manner similar to that in Example 23.

Retention time: 42.8 minutes (1-molecule conjugate)
40.3 minutes (2-molecule conjugate)

Example 51: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTO(2EA)-rhG-CSF Derivative

6.0 mg (25 mol per mol of protein) of the NHS ester (5CHTO(2EA)) obtained in Example 25 was added to 100 µL of the 32 mg/mL rhG-CSF derivative obtained in Reference Example 3 adjusted to 3.8 mg/mL with a 50 mmol/l phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was diluted and 900 µL of the solution was subjected to buffer exchange with a 20 mmol/L acetic acid buffer, whereby 1.3 mL of solution was recovered. This was purified by means of an SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 450 µL of reaction solution containing 335 µg/mL of target substance.(yield: 44.2%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to

that in Example 23 to confirm the bands of 1～3 molecule conjugate.

<Gel Filtration HPLC Analysis>
Analysis was conducted using TSK gel G4000SW$_{XL}$ column in a manner similar to that in Example 23.

Retention time: 42.6 minutes (1-molecule conjugate)
39.5 minutes (2-molecule conjugate)

Example 52: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTM(2EA)-rhG-CSF Derivative

487 mg (48.7 μmol) of the compound in Example 4 was dissolved in sodium chloride, and16.8 mg (146.0 μmol) of NHS and 20.1 mg (145.9 μmol) of DCC were added and ice-cooled for 1 hour following which the mixture was stirred for 2 hours at room temperature under an argon stream. Insoluble material was filtered and the filtrate added to diethyl ether by dripping. The precipitate was recovered and dried under reduced pressure to obtain 260.0 mg of the NHS ester of Compound 4 (yield: 53.4%).

26.6 mg (25 mol per mol of protein) of the NHS ester described above was added to 1.25 mL (4.0 mg/mL) of the rhG-CSF derivative obtained in Reference Example 3 prepared with an isotonic phosphate buffer (pH 7.4), and the mixture was subjected to reaction for an entire day and night at 4°C. A 1.0 mL quantity of reaction solution was subjected to buffer exchange with a 20 mmol/L acetic acid buffer by means of a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.) whereby 1.5 mL of solution was recovered. This was purified by means of an SP-Sepharose F.F. 5.0 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.7 mL of reaction solution containing 1.86 mg/mL of target substance.(yield: 32.5%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

<Gel Filtration HPLC Analysis>
Analysis was conducted using 2 TSK gel G4000SW$_{XL}$ columns in a manner similar to that in Example 23.

Retention time: 48.7 minutes (1-molecule conjugate)
46.9 minutes (2-molecule conjugate)

Example 53: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTM(2EU)-rhG-CSF Derivative

1.0 mg (10 mol per mol of protein) of the compound in Example 5 was added to 50 µL of the rhG-CSF derivative obtained in Reference Example 3 adjusted to 3.9 mg/mL with a 50 mmol/l phosphate buffer (pH 7.3), following which the mixture was subjected to reaction for an entire day and night at 4°C.

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1- and 2-molecule conjugate.

Example 54: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTM(2EA2)-rhG-CSF Derivative

100 mg (0.01 mmol) of the compound in Example 10 was dissolved in 1.0 mL of sodium chloride and 3.5 mg (0.03 mmol) of NHS and 6.2 mg (0.03 mmol) of DCC were added, after which the mixture was placed under an argon stream for 90 minutes at 0°C and following stirring for 2 hours at room temperature the reaction solution was added to diethyl ether by dripping. The white precipitate was dried under reduced pressure to obtain 56.5 mg of the compound of NHS ester from Example 10 (yield: 56.5%).

4.2 mg (10 mol per mol of protein) of the NHS ester described above was added to 210 µL of the rhG-CSF derivative obtained in Reference Example 3 adjusted to 3.9 mg/mL with a 50 mmol/L phosphate buffer (pH 7.5), and the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L acetic acid buffer (pH 4.5) by means of a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.) and, subsequently the solution was purified by means of an SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 965 µL of fraction containing 0.31 mg/mL of target substance.(yield: 39.6%).

<Electrophoresis>

SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

<Gel Filtration HPLC Analysis>

Analysis was conducted using a TSK gel G4000SW_{XL} column in a manner similar to that in Example 23.

Retention time: 42.2 minutes (1-molecule conjugate)
40.6 minutes (2-molecule conjugate)

Example 55: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTM(2URa)-rhG-CSF Derivative

5.2 mg (50 mol per mol of protein) of the compound in Example 8 was added to 50 µL of rhG-CSF derivative obtained in Reference Example 3 adjusted to 3.9 mg/mL with a 50 mmol/l phosphate buffer (pH 7.5), following which 10 µL of 120 mmol/L of sodium borohydride was added and the mixture was subjected to reaction for an entire day and night at 4°C.

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1-molecule conjugate.

Example 56: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5ORN(2UA)-rhG-CSF Derivative

2 mg (10mol per mol of protein) of the compound obtained by activating the compound in Example 13 by means of the method documented in Example 27 was added to 50 µL of rhG-CSF derivative adjusted to 3.7 mg/mL with a 50 mmol/L phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was passed through a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.) equilibrated with a 20 mmol/L acetic acid buffer (pH 4.5), whereby 0.8 mL of solution was recovered. Subsequently the solution was purified by means of an SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 400 µL of reaction solution containing 370 µg/mL target substance (yield: 25.9%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1- and 2-molecule conjugate.

< HPLC Analysis>
Analysis was conducted using two TSK gel G-4000S W_{XL} columns in the manner similar to Example 23.

Retention time: 42.4 minutes (1-molecule conjugate)
39.5 minutes (2-molecule conjugate)

Example 57: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5ORN(2RaA)-rhG-CSF Derivative

2.0 mg (10 mol per mol of protein) of activated PEG derivative (the compound activated by means of the method documented in Example 28 of the compound in Example 14) was added to 50 µL of rhG-CSF derivative adjusted to 3.8 mg/mL with a 50 mmol/l phosphate buffer (pH 7.4), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was passed through a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.) equilibrated with a 20 mmol/L acetic acid buffer (pH 4.5), whereby 0.8 mL of solution was recovered. Subsequently the solution was purified by means of an SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 420 µL of reaction solution containing 71 µg/mL of target substance (yield: 19.9%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1- and 2-molecule conjugate.

< HPLC Analysis>
Analysis was conducted using two TSK gel G4000SW$_{XL}$ columns in the manner similar to Example 23.

Retention time: 42.7 minutes (1-molecule conjugate)
40.8 minutes (2-molecule conjugate)

Example 58: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5DPA(2UA)-rhG-CSF Derivative

2.0 mg (10 mol per mol of protein) of activated PEG derivative (the compound activated by means of the method documented in Example 28 of the compound in Example 14) was added to 50 µL of rhG-CSF derivative adjusted to 3.7 mg/mL with a 50 mmol/l phosphate buffer (pH 7.4), following which the mixture was subjected to

reaction for an entire day and night at 4°C. The reaction solution was passed through a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.) equilibrated with a 20 mmol/L acetic acid buffer (pH 4.5) whereby 0.8 mL of solution was recovered. Subsequently the solution was purified by means of an SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 350 µL of reaction solution containing 67 µg/mL of target substance (yield: 16.0%).

<Electrophoresis>

SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1- and 2-molecule conjugate.

< HPLC Analysis>

Analysis was conducted using two TSK gel G-4000SW_{XL} columns in the manner similar to Example 23.

Retention time: 42.4 minutes (1-molecule conjugate)
39.5 minutes (2-molecule conjugate)

Example 59: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 3-Chain Branched Polyethylene Glycol

Abbreviation: 5SKA(3UA)-rhG-CSF Derivative

16 mg (1.1 µmol) of Compound 5SKA(3UA) in Example 7 was dissolved in 100 µL of sodium chloride and 272 µg of DCC and 152 µg of NHS were added thereto, following which the solution was ice-cooled for 1 hour and then stirred for 1 hour at room temperature mixture. The reaction solution was added to diethyl ether by dripping and the resulting white precipitate was dried under reduced pressure to obtain 14.5 mg of the Compound 5SKA(3UA) NHS ester in Example 7 (yield: 91.0%).

3.6 mg (25 mol per mol of protein) of the NHS ester described above was added to 50 µL of rhG-CSF derivative adjusted to 3.7 mg/mL with a 50 mmol/L phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L acetic acid buffer (pH 4.5) by means of a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.) and the solution was purified by means of an SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.). The target fraction was concentrated to obtain 165 µL of reaction solution containing 0.4 mg/mL of target substance.(yield: 36%).

<Electrophoresis>

SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

< HPLC Analysis>

Analysis was conducted using two TSK gel G4000SW$_{XL}$ columns in the manner similar to Example 23.

Retention time: 42.3 minutes (1-molecule conjugate)
40.2 minutes (2-molecule conjugate)

Example 60: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 4-Chain Branched Polyethylene Glycol

Abbreviation: 5QNA(4UA)-rhG-CSF Derivative

69 mg (3.5 μmol) of Compound 5QNA(4UA) in Example 6 was dissolved in 500 μL of sodium chloride and 1.8 mg of DSC and 0.56 mg of DMAP were added, following which the solution was stirred for 6 hours at room temperature. The reaction solution was added to diethyl ether by dripping and the resulting white precipitate was dried under reduced pressure to obtain 44 mg of the NHS ester of the Compound 5QNA(4UA) in Example 6 (yield: 63%).

5.1 mg (25 mol per mol of protein) of the NHS ester described above was added to 50 μL of the rhG-CSF derivative in Reference Example 3 adjusted to 3.8 mg/mL with a 50 mmol/L phosphate buffer (pH 8), following which the mixture was subjected to reaction for an entire day and night at 4°C.

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1-molecule conjugate.

< HPLC Analysis>
Analysis was conducted using 2 TSK gel G4000SW_{XL} columns in the manner similar to that in Example 23.

Retention time: 40.8 minutes (1-molecule conjugate)

Example 61: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 4-Chain Branched Polyethylene Glycol

Abbreviation: 5PET(3UU)-rhG-CSF Derivative

12.2 mg (10 mol per mol of protein) of the compound obtained in Example 19 was added to 0.5 mL of the rhG-CSF derivative obtained in Reference Example 3 adjusted to 3.1 mg/mL with 20 mmol of a phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L acetic acid buffer by means of a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.). The mixture was purified by means of an SP-Sepharose F.F. 5.0 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.75 mL of reaction solution containing 1.2 mg/mL of target substance.(yield: 58.6%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

< HPLC Analysis>
Analysis was conducted using 2 TSK gel G4000SW$_{XL}$ columns in the manner similar to that in Example 23.

Retention time: 40.5 minutes (1-molecule conjugate)
37.8 minutes (2-molecule conjugate)

Example 62: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 3-Chain Branched Polyethylene Glycol

Abbreviation: 5PET(3UA)-rhG-CSF Derivative

1.6 mg (10 mol per mol of protein) of the NHS ester compound in Example 17 was added to 0.05 mL of the rhG-CSF derivative obtained in Reference Example 36 adjusted to 4.0 mg/mL with 20 mmol of a phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L acetic acid buffer by means of a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.). The mixture was purified by means of an SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.30 mL of reaction solution containing 0.34 mg/mL of target substance.(yield: 56.7).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

<Gel Filtration HPLC Analysis>
Analysis was conducted using 2 TSK gel G4000SW$_{XL}$ columns in the manner similar to that in Example 23.

Retention time: 42.3 minutes (1-molecule conjugate)
39.5 minutes (2-molecule conjugate)

Example 63: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 3-Chain Branched Polyethylene Glycol

Abbreviation: 5SUG(3UA)-rhG-CSF Derivative

2.3 mg of NHS and 4.1 mg of DCC were added to 100 mg (6.7 μmol) of the Compound 5SUG(3UA) prepared in Example 21, following which the solution was ice-cooled for 1 hour then stirred for 1.5 hours at room temperature. The reaction solution was added to diethyl ether by dripping and the resulting white precipitate was dried under reduced pressure to obtain 76.6 mg of the NHS ester of the Compound 5SUG(3UA) in Example 21 (yield: 76.6%).

10.7 mg (35 mol per mol of protein) of the activated compound described above (the NHS ester of the Compound 5SUG(3UA) in Example 21) was added to 0.1 mL of the rhG-CSF derivative obtained in Reference Example 3 adjusted to 3.9 mg/mL with 50 mmol of a phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol/L acetic acid buffer (pH 4.5) by means of a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.). The mixture was purified by means of an SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.39 mL of reaction solution containing 0.28 mg/mL of target substance.(yield: 27.8%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

<Gel Filtration HPLC Analysis>
Analysis was conducted using 2 TSK gel G4000SW$_{XL}$ columns in the manner similar to that in Example 23.

Retention time: 43.0 minutes (1-molecule conjugate)
40.4 minutes (2-molecule conjugate)

Example 64: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 3-Chain Branched Polyethylene Glycol

Abbreviation: 5PET(3URa)-rhG-CSF Derivative

56.3 mg (50 moles per mol of protein) of the compound in Example 20 and 10 μL of sodium cyanoborohydride ($NaBH_3CN$) were added to 0.6 mL of the rhG-CSF derivative adjusted to 2.35 mg/mL with 50 mmol of a phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was acidified by hydrochloric acid and the reaction was halted, after which the reaction

solution was subjected to buffer exchange with a 20 mmol/L acetic acid buffer (pH 4.5). The mixture was purified by means of an SP-Sepharose F.F. 1.4 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.55 mL of reaction solution containing 0.24 mg/mL of target substance.(yield: 8.5%)

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1-molecule conjugate.

<Gel Filtration HPLC Analysis>
Analysis was conducted using 2 TSK gel G4000SW$_{XL}$ columns in the manner similar to that in Example 23.

Retention time: 41.2 minutes (1-molecule conjugate)

Example 65: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 10kDa Linear Chain Branched Polyethylene Glycol

Abbreviation: 10SCM-rhG-CSF Derivative

21.3 mg (4 moles per mol of protein) of the NHS ester compound in Example 22 was added to 2.5 mL of the rhG-CSF derivative obtained in Reference Example 3 adjusted to 4.0 mg/mL with 50 mmol of a phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was passed through a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.) equilibrated with a 20 mmol acetic acid buffer (pH 4.5)whereby 4.0 mL of solution was recovered. This solution was purified by means of an SP-Sepharose F.F. 10.0 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 860 µL of reaction solution containing 2.0 mg/mL of target substance.(yield: 34.4%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

<Gel Filtration HPLC Analysis>
Analysis was conducted using a TSK gel G4000SW$_{XL}$ column in the manner similar to that in Example 23.

Retention time: 42.0 minutes (1-molecule conjugate)
39.5 minutes (2-molecule conjugate)

Example 66: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 20kDa Linear Chain Branched Polyethylene Glycol

Abbreviation: 20SPA-rhG-CSF Derivative

19.1g (4.5 moles per mol of protein) of activated PEG derivative (M-SPA-20,000, Shearwater Polymers, Inc., average molecular weight: 20,000) was added to 995 mL of the rhG-CSF derivative prepared to 4.0 mg/mL with 50 mmol of a phosphate buffer (pH 7.6), following which the mixture was subjected to reaction for an entire day and night at 4°C. Subsequently, purification was effected by passing the solution through an SP-Sepharose F.F. 2000 mL column (Amersham-Pharmacia Biotech, Inc.) equilibrated with a 20 mmol acetic acid buffer (pH 4.5). A quantity of 4000 mL of target fraction was concentrated to obtain 320 mL of reaction solution containing 11.2 mg/mL of target substance.(yield: 90.4%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

<Gel Filtration HPLC Analysis>
Analysis was conducted using 2 TSK gel G-4000SW$_{XL}$ columns in the manner similar to that in Example 23.

Retention time: 38.2 minutes (1-molecule conjugate)
34.4 minutes (2-molecule conjugate)
32.2 minutes (3-molecule conjugate)

Example 67: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 2- Chain Branched Polyethylene Glycol

Abbreviation: PEG$_2$Lys-rhG-CSF Derivative

10.6 mg (10 moles per mol of protein) of PEG2Lys (average molecular weight: 10,000, Shearwater Polymers, Inc.,) was added to 0.5 mL of the rhG-CSF derivative obtained in Reference Example 3 prepared to 4.0 mg/mL with 50 mmol of a phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with a 20 mmol sodium acetic acid buffer (pH 4.5). This solution was purified by means of an SP-Sepharose F.F. 2.0 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.5 mL of reaction solution containing 1.05 mg/mL of target substance (yield: 26.3%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

<Gel Filtration HPLC Analysis>
Analysis was conducted using 2 TSK gel G4000SW$_{XL}$ columns in the manner similar to that in Example 23.

Retention time: 44.3 minutes (1-molecule conjugate)
41.7 minutes (2-molecule conjugate)

Example 68: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 2- Chain Branched Polyethylene Glycol

Abbreviation: 5CHTM(2EA)-rhG-CSF Derivative

28.0 mg (15 moles per mol of protein) of the 5CHTM(2EA) NHS ester obtained in Example 26 was added to 0.9 mL of the rhG-CSF derivative in Reference Example 3 prepared to 3.9 mg/mL with an isotonic phosphate buffer (pH 7.4), following which the mixture was subjected to reaction for an entire day and night at 4°C.

A quantity of 0.8 mL of reaction solution was subjected to buffer exchange with a 20 mmol acetic acid buffer (pH 4.5) by means of a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc) whereby 1.5 mL of solution was recovered. This solution was purified by means of an SP-Sepharose F.F. 5.0 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 1.0 mL of reaction solution containing 1.3 mg/mL of target substance (yield: 33.0%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

<Gel Filtration HPLC Analysis>
Analysis was conducted using 2 TSK gel G4000SW$_{XL}$ columns in the manner similar to that in Example 23.

Retention time: 42.8 minutes (1-molecule conjugate)
40.1 minutes (2-molecule conjugate)

Example 69: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with a 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTC(2AA)-rhG-CSF

10.0 mg (25 moles per mol of protein) of the 5CHTC(2AA) NHS ester obtained in Example 24 was added to 0.2 mL of the rhG-CSF solution obtained in Reference Example 4 adjusted to 3.9 mg/mL with an isotonic phosphate buffer (pH 7.4), following which the mixture was subjected to reaction for an entire day and night at 4°C.

A quantity of 0.2 mL of reaction solution was subjected to buffer exchange with a 20 mmol acetic acid buffer (pH 4.5) by means of a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc) whereby 1.0 mL of solution was recovered. This solution was purified by means of an SP-Sepharose F.F. 1.0 mL column (Amersham-Pharmacia Biotech, Inc.) to obtain 0.3 mL of reaction solution containing 0.7 mg/mL of target substance (yield: 26.8%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1～3 molecule conjugate.

< HPLC Analysis>
Analysis was conducted using two TSK gel G4000 W_{XL} columns in a manner similar to that in Example 23.

Retention time: 42.9 minutes (1-molecule conjugate)
40.4 minutes (2-molecule conjugate)

Example 70: Preparation of Recombinant Human Granulocyte-colony stimulating factor modified with a 5kDa Three-Chain Branched Polyethylene Glycol

Abbreviation: 5SKA(3UA)-rhG-CSF

16 mg (1.1 μmol) of the compound of Example 7 (5SKA(3UA)) was dissolved in 100 μL of methylene chloride, and 272 μg of DCC and 152 μg of NHS were added, followed by stirring under ice-cooling for one hour and at room temperature for one hour. The reaction mixture was added to diethyl ether by dripping, and the formed white precipitate was dried under reduced pressure to obtain 14.5 mg of the NHS ester of the compound of Example 7 (yield: 91%).

12.2 mg (25 moles per mol of protein) of the compound activated above (NHS ester) was added to 140 µL of a 4.4 mg/mL solution of the rhG-CSF derivative obtained in Reference Example 4 in 50 mmol/l phosphate buffer (pH 7.5), and the mixture was subjected to reaction an entire day and night at 4°C. The reaction mixture was applied to a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.) and subjected to buffer exchange with 20 mmol/l acetic acid (pH 4.5), followed by purification using an SP Sepharose F.F. column (1.8 mL, Amersham-Pharmacia Biotech, Inc.).

The target fraction was concentrated to obtain 110 µL of a solution containing the target product (1.1 mg/mL) (yield: 19%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1- to 3-molecule conjugates.

<Gel Filtration HPLC Analysis>
Analysis was conducted using two TSK gel G4000W_{XL} columns in a manner similar to Example 23.

Retention time: 40～45 minutes (1～3 molecule conjugate)

Example 71: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative Modified with 5KDa Linear Chain Branched Polyethylene Glycol

Abbreviation: PEG_2Lys-rhG-CSF

11.7 mg (10 moles per mol of protein) of PEG_2Lys (Shearwater Polymers, Inc.) was added to 0.5 mL of an rhG-CSF solution obtained in Reference Example 4 prepared to 4.4 mg/mL with an isotonic phosphate buffer (pH 7.4), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was applied to a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.) and subjected to buffer exchange with a 20 mmol/l acetic acid (pH 4.5). The mixture was purified with an SP-Sepharose F.F. column of 2.0 mL (Amersham-Pharmacia Biotech, Inc.) to obtain 0.5 mL of reaction solution containing 1.78 mg/mL of target substance (yield: 40.5%).

<Electrophoresis>
SDS-PAGE was carried out in the absence of 2-mercaptoethanol in a manner similar to that in Example 23 to confirm the bands of 1- to 3-molecule conjugates.

<Gel Filtration HPLC Analysis>
Analysis was conducted using two TSK gel G4000SW_{XL} columns in a manner similar to Example 23.

Retention time: 44.2 minutes (1-molecule conjugate)
41.8 minutes (2-molecule conjugate)

Example 72: Preparation of bovine Cu, Zn-Superoxide Dismutase modified with 5kDa two-chain branched polyethylene glycol

Abbreviation: 5CHTC(2AA)-bSOD

30 mg (30 μmol) of the compound in Example 2 was dissolved in 1 mL of methylene chloride, and 1.7 mg (0.015 mmol) of NHS and 3.1 mg (0.015 mmol) of DCC were added, after which the mixture was stirred for 30 minutes at O°C. The white precipitate was dried under reduced pressure to obtain 21 mg of NHS ester of the compound of Example 2 (yield: 70%).

10 μL (50 moles per mole of protein) of an aqueous solution (156 mg/mL distilled water) of the previously described NHS ester obtained earlier was added to 50 μL of bovine Cu, Zn SOD solution (2 mg/mL, pH 9 boric acid buffer, Wako Pharmaceuticals), following which the mixture was subjected to reaction for an entire day and night at 4°C.

<Electrophoresis>
SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1- and 2-molecule conjugates.

Example 73: Preparation of Bovine Cu, Zn-Superoxide Dismutase Modified with 5kDa Double-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTO(2EA)-bSOD

A quantity of 20 mg of the compound in Example 3 was activated under conditions similar to those described in Example 25, whereby 13 mg of the NHS ester compound of Example 3 was obtained (yield: 65%).

10 μL (50 moles per mole of protein) of an aqueous solution (156 mg/mL distilled water) of the previously described NHS ester obtained earlier was added to 50 μL of bovine Cu, Zn SOD solution (2 mg/mL, pH 9 boric acid buffer, Wako Pharmaceuticals), following which the mixture was subjected to reaction for an entire day and night at 4°C.

<Electrophoresis>
SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1- and 2-molecule conjugates.

Example 74: Preparation of Bovine Cu, Zn-Superoxide Dismutase Modified with 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTO(2UU)-bSOD

10 μL (50 moles per mole of protein) of an aqueous solution (156 mg/mL distilled water) of the compound obtained in Example 1 was added to 50 μL of bovine Cu, Zn SOD solution (2 mg/mL, pH 9 boric acid buffer, Wako

Pharmaceuticals), following which the mixture was subjected to reaction for an entire day and night at 4°C.

<Electrophoresis>
SDS-PAGE was carried out under conditions similar to those described in Example 23 to confirm the bands of 1- to 3-molecule conjugates.

Example 75: Preparation of Bovine Cu, Zn-Superoxide Dismutase Modified with 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTM(2EA)-bSOD

A quantity of 487 mg (48.7 µmol) of the compound in Example 4 was activated using the same method and under the same conditions as those described in Example 26, whereby 260 mg of the NHS ester compound of Example 4 was obtained (yield: 53.4%).

10 µL (50 moles per mole of protein) of an aqueous solution (156 mg/mL distilled water) of the previously described NHS ester compound previously prepared to 50 µL was added to 50 µL of bovine Cu, Zn SOD solution (2 mg/mL, pH 9 boric acid buffer, Wako Pharmaceuticals), whereby the mixture was subjected to reaction for an entire day and night at 4°C.

<Electrophoresis>
SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1- to 3-molecule conjugates.

Example 76: Purification of Bovine Cu, Zn-Superoxide Dismutase Modified with 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTM(2EA)-bSOD (Purified)

A quantity of 360 mg (36.0 µmol) of the compound in Example 4 was activated under the same conditions and with the same method indicated in Example 26 to obtain 181.9 mg of the NHS ester compound in Example 4 (yield: 50.5%).

33.9 mg (25 moles per mole of protein) of the NHS ester compound in Example 4 was added to 2.2 mL of bovine Cu, Zn SOD solution (2 mg/mL, pH 9 boric acid buffer, Wako Pharmaceuticals), following which the mixture was subjected to reaction for an
entire day and night at 4°C. Subsequently, the reaction solution was purified by means of an SP-Sepharose F. F. 4.3 mL column (Amersham-Pharmacia Biotech, Inc.).

The target fraction excluding the unmodified SOD was concentrated to obtain 200 µL of reaction solution containing 3.73 mg/mL of target product (yield: 17.2%). Furthermore, activity was restored by adding to the $CuSO4_2$ and $ZnSO_4$ aqueous solutions to produce 10 mmol/L of each.

<Electrophoresis>
SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1- to 3-molecule conjugates.

Example 77: Preparation of Bovine Cu, Zn-Superoxide Dismutase Modified with 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5ORN(2UA)-bSOD

A quantity of 20 mg of the compound in Example 13 was activated under the same conditions and with the same method indicated in Example 27 whereby 16.0 mg of the NHS ester compound in Example 13 was obtained (yield: 80.0%).

10 µL (50 moles per mole of protein) of an aqueous solution (156 mg/mL distilled water) of the NHS ester previously activated was added to 50 µL of bovine Cu, Zn SOD solution (2.0 mg/mL, pH 9 boric acid buffer, Wako Pharmaceuticals), following which the mixture was subjected to reaction for an entire day and night at 4°C.

<Electrophoresis>
SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1- to 5-molecule conjugates. However, unmodified SOD was not detected.

Example 78: Preparation of Bovine Cu, Zn-Superoxide Dismutase Modified with 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5DPA(2UA)-bSOD

A quantity of 20 mg of the compound in Example 15 was activated under the same conditions and with the same method previously indicated in Example 29 whereby 12.0 mg of the NHS ester compound of Example 15 was obtained (yield: 60.0%).

10 µL (50 moles per mole of protein) of an aqueous solution of (156 mg/mL distilled water) of the previously described NHS ester solution was added to 50 µL of bovine Cu, Zn SOD solution (2.0 mg/mL, pH 9 boric acid buffer, Wako Pharmaceutical), following which the mixture was subjected to reaction for an entire day and night at 4°C.

<Electrophoresis>
SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1- to 5-molecule conjugates. However, unmodified SOD was not detected.

Example 79: Preparation of Bovine Cu, Zn-Superoxide Dismutase Modified with 10kDa Linear Chain Branched Polyethylene Glycol

Abbreviation: 10SCM-bSOD (Purified)

18.8 mg (15 moles per mole of protein) of the NHS ester compound in Example 22 (prepared as in Example 40) was added to 1.0 mL of bovine Cu, Zn-SOD solution (2.0 mg/mL, 50 mmol/L boric acid buffer pH 9.0, Wako Pharmaceutical), following which the mixture was subjected to reaction for an entire day and night at 4°C.

Subsequently, the reaction solution was purified by means of an SP-Sepharose F. F. 2.0 mL column (Amersham-Pharmacia Biotech, Inc.) following which the target fraction excluding unmodified bSOD was concentrated to obtain 120 µL of 5.9 mg/mL reaction solution. Furthermore, activity was restored by adding to the $CuSO4_2$ and $ZnSO_4$ aqueous solutions to produce 10 mmol/L of each.

<Electrophoresis>
SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1- to 5-molecule conjugates.

Example 80: Preparation of Human Cu, Zn-Superoxide Dismutase Modified with 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTM(2EA)-hSOD

A quantity of 487 mg (48.7 µmol) of the compound in Example 4 was activated under the same conditions and with the same method previously indicated in Example 26, whereby 260 mg of the NHS ester compound of Example 4 was obtained (yield: 53.4%).

10 µL (50 moles per mole of protein) of an aqueous solution (156 mg/mL distilled water) of the previously described aqueous NHS ester solution previously prepared to 50 µL was added to 50 µL of human Cu, Zn SOD solution (1.9 mg/mL, pH 9 boric acid buffer, Cellular Products, Inc.), following which the mixture was allowed to stand for an entire day and night at 4°C.

<Electrophoresis>

SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1- to 3-molecule conjugates.

Example 81: Preparation of Human Cu, Zn-Superoxide Dismutase Modified with 5kDa 2-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTM(2UM)-hSOD (Purified)

3.13 mg (10 moles per mole of protein) of the compound in Example 9 was added to 0.6 mL of a solution of human Cu, Zn SOD [2.63 mg/mL, in a phosphate buffer (pH 7.5), Cellular Products, Inc.], following which the mixture was allowed to stand for an entire day and night at 4°C.

Subsequently, the mixture was purified using a Sephacryl S-300 gel filtration 20 mL column (Amersham-Pharmacia Biotech). The modified fraction excluding the unreacted SOD was recovered and concentrated to 0.5 mL. This solution was subjected to buffer exchange with 20 mmol/L acetic acid (pH 3.5) by means of a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.), whereby 0.8 mL of solution was recovered. This solution was purified using a SP-Sepharose F.F. column (0.7 mL, Amersham-Pharmacia Biotech, Inc.), and concentrated to the target fraction. Furthermore, SOD activity was restored by adding to the $CuSO4_2$ and $ZnSO_4$ aqueous solutions to produce 10 mmol/L of each. A quantity of 180 µL of solution containing 0.25 mg/mL of target product was obtained (yield: 4.5%).

<Electrophoresis>
SDS-PAGE was carried out under the same conditions as in Example 23 to confirm the band of 1-molecule conjugate.

Example 82: Preparation of Human Cu, Zn-Superoxide Dismutase Modified with 5kDa 3-Chain Branched Polyethylene Glycol

Abbreviation: 5PET(3UM)-hSOD (Purified)

3.1 mg (10 moles per mole of protein) of the compound obtained in Example 18 was added to 0.5 mL (1.34 mg/mL) Cu, Zn-hSOD solutions (Cellular Products, Inc.) prepared with a 50 mmol/L phosphate buffer (pH 7.5), following which the mixture was subjected to reaction for an entire day and night at 4°C. The reaction solution was subjected to buffer exchange with 20 mmol/L sodium acetic acid (pH 3.5) by means of a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.). This solution was purified by means of a SP-Sepharose F.F. 0.7 mL column (Amersham-Pharmacia Biotech, Inc.), whereby 0.62 mL of solution containing 0.33 mg/mL of target product was obtained (yield: 30.6%).

<Electrophoresis>
SDS-PAGE was carried out in a manner similar to that in Example 23 in the absence of 2-mercaptoethanol to confirm the bands of 1-molecule conjugates.

<Gel Filtration HPLC Analysis>
Analysis was conducted using two TSK gel G4000SW$_{XL}$ columns in a manner similar to that in Example 23.

Retention time: 41.1 minutes (1-molecule conjugate)

Example 83: Preparation of Anti-GD3 Chimera Antibody Modified with 5kDa Two-Chain Branched Polyethylene Glycol

Abbreviation: 5CHTM(2EA)-KM-871

1.0 mg (10 moles per 1 mole of protein) of the NHS ester compound obtained in Example 4 was added to 0.5 mL of 2.6 mg/mL KM-871 prepared solution using a 20 mmol/L phosphate buffer (pH 7.5) (prepared according to Japanese Published Unexamined Patent Application No. H05-304989), following which the mixture was subjected to reaction for an entire day and night at 4°C. A quantity of 0.5 mL of the reaction solution was subjected to buffer exchange with a 20 mmol/L sodium acetic acid (pH 4.5) by means of a Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.), whereby 0.8 mL of solution was recovered. This solution was purified using a CM-Sepharose F.F. 1.2 mL column (Amersham-Pharmacia Biotech, Inc.), whereby 0.38 mL of solution containing 0.59 mg/mL of the target product was obtained (yield: 17.1%).

<Electrophoresis>
SDS-PAGE was carried out in a manner similar to that in Example 23 to confirm the bands of 1- and 2-molecule conjugates.

Example 84: Preparation of Anti-GD3 Chimera Antibody Modified with 5kDa 3-Chain Branched Polyethylene Glycol

Abbreviation: 5PET(3UA)-KM-871

0.6 mg (5 moles per 1 mole of protein) of the 5PET(3UA) NHS ester obtained in Example 36 was added to 1.0 mL of 1.1 mg/mL KM-871 prepared solution using a 20 mmol/L phosphate buffer (pH 7.5) (prepared according to Japanese Published Unexamined Patent Application No. H05-304989), following which the mixture was subjected to reaction for an entire day and night at 4°C. A quantity of 1.0 mL of reaction solution was subjected to buffer exchange with a 20 mmol/L acetic acid buffer (pH 4.5) using Sephadex G-25 column (Amersham-Pharmacia Biotech, Inc.). This solution was

purified using a CM-Sepharose F.F. 1.0 mL column (Amersham-Pharmacia Biotech, Inc.), whereby 430 µL of solution containing 0.52 mg/mL of target product was obtained (yield: 20.4%).

<Electrophoresis>
SDS-PAGE was carried out with the same method of Example 23 in the absence of 2-mercaptoethanol to confirm the bands of 1- and 2-molecule conjugates.

Test Example 1: Antiviral Activity of Chemically Modified Interferon-ß

The antiviral activity of the chemically modified rhIFN-ß obtained in Examples 23~43, chemically modified natural hIFN-ß, unmodified rhIFN-ß, and unmodified natural hIFN-ß was examined using the following neutral red (NR) uptake method.

<NR Uptake Method>
Antiviral activity was measured by referring to the method of Kohase, et al. [Protein,Nucleic Acid and Enzyme (separate volume), p. 355 (1981)].

Namely Eagle MEM culture supplemented with 5% fetal bovine serum (FBS) was added to a sterilized transfer plate. Next, 50 µL each of solutions of domestic standard IFN preparations [α (The Green Cross Corporation) and ß (Toray Industries, Inc.)] were put into wells, followed by 2-fold serial dilution. Elsewhere 50 µL each of chemically modified IFNs or unmodified IFNs diluted with a medium to predetermined concentrations were put into wells. These IFN solutions were transferred to a 96-well plate containing a predetermined number of cells of an established cell line (FL cell) derived from human amnion, followed by stirring for several seconds. The resulting mixtures were incubated an entire day and night in a CO_2 incubator at 37° C to induce an antiviral state.

Next, the culture solutions were removed, and a virus solution was added, followed by incubation in a CO_2 incubator at 37°C for 2 days to effect viral infection. The antiviral state of the cells was changed by IFN, and cell degeneration occurred. Subsequently, the culture solutions were removed, and a neutral red (NR) solution was added. The plate was allowed to stand in a CO_2 incubator at 37°C for one hour, followed by removal of the NR solution. After the wells were washed with an isotonic phosphate buffer, an extracting liquid (0.01 mol/l hydrochloric acid--30% ethanol) was added, followed by stirring for 2 to 3 minutes.

The surviving cells were stained with NR. After extraction, the absorbance at 492 nm was measured, and a standard curve was plotted. The relative activity of each chemically modified IFN was calculated based on the activity of the unmodified IFN calculated from the standard curve which was defined as 100%.

The relative activity of each IFN-β is shown in Tables 1, 2 and 3.

TABLE 1 Antiviral Activity of Chemically Modified Recombinant Human Interferon-β

Compound Abbreviation	Example No.	Relative Activity %
Unmodified rhIFN-β	-	100
5CHTO(2UU)-rhIFN-β	23	96
5CHTC(2AA)-rhIFN-β	24	122
5CHTO(2EA)-rhIFN-β	25	90
5CHTM(2EA)-rhIFN-β	26	116
5TRC(3UA)-rhIFN-β	33	58
5SKA(3UA)-rhIFN-β	34	93
5PET(3UA)-rhIFN-β	36	50
PEG_2Mal-rhIFN-β	30	78
20SPA-rhIFN-β	38	>100
5SPA-rhIFN-β	39	>100
20Mal-rhIFN-β	31	60
5Mal-rhIFN-β	32	80
5ORN(2UA)-rhIFN-β	27	>100
5ORN(2RaA)-rhIFN-β	28	>100
5DPA(2UA)-rhIFN-β	29	88

TABLE 2 Antiviral Activity of Chemically Modified Recombinant Human ^{17}Ser IFN-β

Compound Abbreviation	Example No.	Relative Activity %
Unmodified-^{17}Ser IFN-β	-	100
5CHTM(2EA)-^{17}Ser IFN-β	42	70
5PET(3UA)-^{17}Ser IFN-β	43	115

TABLE 3 Antiviral Activity of Chemically Modified Natural Human Interferon-β

Compound Abbreviation	Example No.	Relative Activity %
Unmodified Natural hIFN-β	-	100
5CHTM(2EA) Natural hIFN-β	41	104

It was confirmed by the results above that all the chemically modified IFN-β employed with the present invention retained antiviral activity.

Test Example 2: Antiviral Activity of Chemically Modified Interferon-α

The antiviral activity of chemically modified rhIFN-α obtained in Example 44 to Example 46 and that of unmodified rhIFN-α were examined by the NR uptake method illustrated in Test Example 1.

The antiviral activity of each IFN-α used at a concentration of 1 μg/mL was retained in its entirety by each chemically modified IFN-α (5CHTC(2AA)-IFN-α in Example 44, 5CHTM(2EA) in Example 45, and 5PET(3UA)-IFN-α in Example 46) (indicated as a relative activity based on the activity of unmodified IFN-α).

Test Example 3: Enzyme Activity of Chemically Modified Superoxide Dismutase

The enzyme activity of the chemically modified SOD prepared in Examples 72～82 was measured by the xanthine-xanthine oxidase-cytochrome C system of Mccord, J. M. and Fridovichi, I. [J. Biol. Chem., Vol. 244, p. 6049 (1969)]. One unit (U) of SOD activity is an enzyme amount of SOD which inhibits the reducing rate of cytochrome C by 50% at 30°C and a pH of 7.8 and was calculated according to the following equation:

$$\text{Specific Activity (U/mg)} = [(\text{blank} / \Delta A / \text{min} - 1] \times 1 / 0.000256$$

The enzyme activities of chemically modified bovine SOD and chemically modified human SOD are shown in Tables 4 and 5, respectively.

SOD 50 U/mL = 0.000256 mg (at 3900U/mg)

ΔA/min.: Measured value

TABLE 4 Enzyme Activity of Chemically Modified Bovine Cu, Zn Superoxide Dismutase

Compound Abbreviation	Example No.	Relative Activity %
Unmodified bSOD	-	100
5CHTC(2AA)-bSOD	72	72
5CHTM(2EA)-bSOD	75	90
5CHTM(2EA)-bSOD (Purified)	76	114
5ORN(2UA)-bSOD	77	82
5DPA(2UA)-bSOD	78	77

The activity was indicated as a relative activity based on the enzyme activity of unmodified bSOD defined as 100%.

TABLE 5 Enzyme Activity of Chemically Modified Human Cu, Zn Superoxide Dismutase

Compound Abbreviation	Example No.	Relative Activity %
Unmodified hSOD	-	100
5CHTM(2EA)-hSOD	80	101
5CHTM(2UM)-hSOD (Purified)	81	92
5PET(3UM)-hSOD (Purified)	82	50

The activity was indicated as a relative activity based on the enzyme activity of unmodified hSOD defined as 100%.

It was confirmed that chemically modified hSOD used with the present invention retained enzyme activity.

Test Example 4 Growth-Promoting Activity of Chemically Modified Recombinant Human Granulocyte-Colony Stimulating Factor Derivative on Mouse Leukemia Cell NFS60

The growth-promoting activity of the compounds of Examples 49 ~71 by unmodified rhG-CSF derivative and unmodified rhG-CSF on mouse leukemia cell NFS60 [Proc. Natl. Acad. Sci USA, Vol. 82, p. 6687 (1985)] was measured according to the method of Asano, et al. [Yakuri To Chiryō, Vol. 19, p. 2767 (1991)].

NFS60 cell growth-promoting activity of unmodified rhG-CSF derivatives (affected in cells with a 100ng/mL concentration) was recognized in affected cells at a 100ng/mL concentration in Examples 49 ~ 63 and in Example 66 (the activity retained was equivalent to an unmodified rhG-CSF derivative).

NFS60 cell growth-promoting activity of unmodified rhG-CSF (activity in cells with a 100ng/mL concentration) was recognized in affected cells with a 100ng/mL concentration in Examples 68 ~ 70 (The activity retained was equivalent to unmodified rhG-CSF).

It was confirmed that in the present invention the cell growth-promoting effect of NFS60 cells in chemically modified rhG-CSF derivatives and chemically modified rhG-CSF used was maintained.

Test Example 5 Binding Activity of Chemically Modified Anti-GD3 Chimera Antibody

The binding activity of the chemically modified anti-GD3 chimera antibody prepared in Example 84 was measured according to the method of Kenya. S, et al. [Cancer Immunol. Immunother., Vol. 36, p. 373 (1993)].

The GD3 binding activity of the chemically modified anti-GD3 chimera antibody is shown in Table 6.

The activity was indicated as a relative activity based on the binding activity of unmodified anti-GD3 chimera antibody defined as 100%.

TABLE 6 GD3-Binding Activity of Chemically Modified Antibody

Compound Abbreviation	Example No.	Relative Activity %
Unmodified Antibody	-	100
5PET(3UA)-KM-871	84	20

It was confirmed that the chemically modified anti-GD3 chimera antibody in the present invention retained GD3-binding activity.

Test Example 6 Results of Macrogol Ointment Stability of Chemically Modified Recombinant Human Interferon-β

Solutions of polyethylene glycol modified rhIFN-β, unmodified rhIFN-β, and an unmodified rhIFN-β solution to which 2 mg/mL mPEG (average molecular weight: 20,000) was added were each prepared using a 20 mmol phosphate buffer (pH 6.0) containing ethylene glycol and sodium chloride in a manner by which a 1.0 mg/mL concentration was attained as described in Example 26. The ointments were prepared by adding 100 μL of each solution to 0.9g of Macrogol ointment (Toho Pharmaceuticals Co.) and kneading the mixture for 5 minutes to a homogeneous consistency. A quantity of 100 mg of each ointment was dispensed into an Eppendorf tube and stored sealed at room temperature. The ointment was sampled 24 hours after its preparation and then dissolved in a 20 mmol/L phosphoric acid buffer (pH 6.0) containing ethylene glycol and sodium chloride, following which the antiviral activity of the mixture was measured.

The activity after 24 hours is shown in Table 7 as a relative activity based on the activity immediately after preparation of the ointment defined as 100%.

A reduction in activity was confirmed in the unmodified rhIFN-β and unmodified rhIFN-β to which mPEG had been added, but activity was retained in the chemically modified rhIFN-β solution.

TABLE 7 Change in Activity Due to Presence of rhIFN-β in Ointment

Compound Abbreviation	Immediately After Preparation	24 Hours Later
Unmodified rhIFN-β	100%	89.9%
Unmodified rhIFN-β with mPEG added	100%	72.5%
5CHTM(2EA) rhIFN-β	100%	112.3%

TEST Example 7 Stability Results of a Hydrophilic Ointment with Chemically Modified Recombinant Human Interferon-β

Solutions of polyethylene glycol modified rhIFN-β, unmodified rhIFN-β, and an unmodified rhIFN-β solution to which 2 mg/mL of mPEG (average molecular weight: 20,000) was added were each prepared using a 20 mmol phosphate buffer (pH 6.0) containing ethylene glycol and sodium chloride in a manner by which a 1.0 mg/mL concentration was attained as described in Example 26. The ointments were prepared by adding 100 μL of each solution to 0.9g of Macrogol ointment (Iwaki Seiyaku Co., Ltd.) and kneading the mixture for 5 minutes to a homogeneous consistency. A quantity of 100 mg of each ointment was dispensed into an Eppendorf tube and stored sealed at 37°C. The ointments were sampled periodically over time and then suspended in a 20 mmol/L phosphoric acid buffer (pH 6.0) containing ethylene glycol and sodium chloride.

Calculations were gained from the standard curve provided by ELISA of the weight of rhIFN-β in the suspended solution.

Results are shown in Figure 1. The concentrations of both the chemically modified rhIFN-β and the unmodified rhIFN-β had gradually decreased; however the residual ratio of the chemically modified rhIFN-β was higher than that of the unmodified rhIFN-β.

Test Example 8 Stability Results of Macrogol Ointment with a Chemically Modified Bovine Cu, Zn-Superoxide Dismutase

Solutions of 5CHTM(2EA)-bSOD (purified) prepared to a concentration of 3.73 mg/mL (as prepared in Example 76), unmodified bSOD, and unmodified bSOD with mPEG added thereto were each prepared using a 20 mmol acetic acid buffer (pH 4.5), and homogeneous ointments were prepared by adding 50 µL of each solution to 450 mg of Macrogol ointment. . Next, a quantity of 50 mg from each ointment was extracted over time and 200 µL of an isotonic phosphate buffer was added, following which part of the solution was used to measure the activity.

The changes in activity of the extracted quantities over time are shown in Figure 2, based on the bSOD activity immediately following ointment preparation defined as 100%. There was a reduction in the activity of the ointment with respect to time; however, only the PEG-modified 5CHTM(2EA)-bSOD retained high activity over a prolonged period of time.

Test Example 9 Stability Results of Macrogol Ointment with a Chemically Modified Recombinant Human Granulocyte-Colony Stimulating Factor Derivative

To 0.9g of Macrogol ointment (Toho Pharmaceuticals Co.) were added 100 µL quantities of a derivative solution prepared with a 6 mg/mL concentration using an acetic acid buffer (pH 4.5) as prepared in Example 66 (20SPA-rhG-CSFderivative), an unmodified rhG-CSF derivative solution with the same concentration, and an rhG-CSF derivative solution with the same concentration to which was added a 12 mg/mL PEG (average molecular weight: 20,000, Nippon Oil and Fats, Ltd.), whereby ointments were prepared having each been kneaded for 5 minutes to a homogeneous consistency. A 100 mg quantity of each ointment was dispensed into an Eppendorf tube and stored sealed at room temperature. Each tube was sampled over time, and the ointments added thereto with a 20 mmol/L acetic acid buffer containing 150 mmol of sodium chloride were dissolved. The supernatant of this solution was analyzed by electrophoresis under the same conditions as those described in Example 23, following which changes in the protein bands were observed.

As shown in Figures 3 and 4, no changes were observed even after 72 hours in the bands of the chemically modified rhG-CSF derivative, where at 24 hours after preparation the ointment with unmodified rhG-CSF derivative the band had become thin, and at 72 hours it had disappeared almost completely.

The above results suggest that the chemically modified rhG-CSF derivative is present stably within the Macrogol ointment but that the unmodified rhG-CSF derivative gradually changes and forms a precipitate.

Test Example 10: Stability Results of a Hydrophilic Ointment Containing a Chemically Modified Recombinant Human Granulocyte-Colony Stimulating Factor Derivative

To 0.9g of a hydrophilic ointment (Iwaki Seiyaku Co., Ltd.) was added 100 µL each of a derivative solution prepared as in Example 66 with a 6 mg/mL concentration using an acetic acid buffer (pH 4.5) (20SPA-rhG-CSF derivative) and of an unmodified rhG-CSF derivative solution with the same concentration whereby ointments were prepared having each been kneaded to a homogeneous consistency for 5 minutes. A 100 mg quantity of each ointment was dispensed into an Eppendorf tube and stored sealed at 37°C. Each tube was sampled over time, and 20 mmol/L of an acetic acid buffer containing 150 mmol of sodium chloride was added and the ointments were suspended. The suspended solution had been suspended in a manner whereby it would become homogeneous but was then separated by centrifuge at 15,000rpms for 20 minutes. The aqueous layer (lower layer) was recovered and analyzed by electrophoresis using the method described in Example 23.

Figures 5 and 6 show the changes taking place over time in the ointments stored at 37°C .

Though no changes were observed in the band of the chemically modified rhG-CSG derivative after 24 hours, the band of the unmodified rhG-CSF derivative gradually began thinning starting immediately after ointment preparation and after 2 hours it had disappeared almost completely.

Figures 7 and 8 show the changes taking place over time in the ointments stored at room temperature.

Though no remarkable changes were observed in the band with the chemically modified rhG-CSF derivative after 30 days, the band of the unmodified rhG-CSF derivative became gradually thinner and had disappeared almost completely after 30 days. Even in the ointment that had been prepared with 100 μL of an acetic acid buffer (pH 4.5) containing an unmodified rhG-CSF derivative solution with a single mPEG (12 mg/mL mPEG, average molecular weight: 20,000) and a 6 mg/mL unmodified rhG-CSG derivative, disappearance of the band was not suppressed.

The above results suggest that the hydrophilic ointment containing the chemically modified rhG-CSF derivative is present stably but that the extant precipitate in the ointment containing the unmodified rhG-CSF derivative gradually changes.

Reference Example 1: Preparation of Recombinant Human Interferon-β (Unmodified rhIFN-β)

rhIFN-β was produced according to the method of Mizukami, et al. [Biotechnology Letter, Vol. 8, p. 605 (1986)] and the method of Kuga, et al. [Gendai Kagaku (Chemistry Today), special number 12: Igaku Ni Okeru Idenshi Kogaku (Gene Engineering in Medical Science), p. 135 (1986), Tokyo Kagaku Dojin (Tokyo Chemistry Magazine)].

Escherichia coli K-12 carrying plasmid pMG-1 comprising DNA encoding rhIFN-β was seed-cultured in LGTrpAp medium (10 g/l bactotrypton, 5 g/l yeast extract, 5 g/l sodium chloride, 1 g/l glucose, 50 mg/l L-tryptophan and 50 μg/l ampicillin). For the production of rhIFN-β, culturing was carried out in a 2L jar fermenter using MCGAp medium (a medium prepared by adding 0.5% casamino acid and 50 μg/mL ampicillin to M9 medium) and culturing for several days at 20°C with the glucose concentration maintained at 1% and the pH at 6.5%.. The culture was shaken at 750 rpm and aerated at 1 L/minute. From the culture, an extract was prepared by the freezing and thawing method [DNA, Vol. 2, p. 265 (1983)]. Further, rhIFN-β was obtained from the cell residue according to the method disclosed in Japanese Published Unexamined Patent Application No. S61-69799.

Reference Example 2: Preparation of Recombinant Human Interferon-γ

rhIFN-γ was prepared using the rhIFN-β production method of Ito, et al [Ikabunshi Seibutsu Gaku (Biomolecular Science) p. 355 (1987), Minami Publishing] and of Kuga, et al. [Gendai Kagaku (Chemistry Today), special number 12: Igaku Ni Okeru Idenshi Kogaku (Gene Engineering in Medical Science), p. 135 (1986), Tokyo Kagaku Dojin (Tokyo Chemistry Magazine)].

Escherichia coli pGKA2 carrying plasmid pKYP10 comprising DNA encoding rhIFN-γ was seed-cultured in LGTrpAp medium (10 g/l bactotrypton, 5 g/l yeast extract, 5 g/l sodium chloride, 1 g/l glucose, 50 mg/l L-tryptophan and 50 μg/l ampicillin). For the production of rhIFN-γ, culturing was carried out in a 2L jar fermenter using MCGAp medium (a medium prepared by adding 0.5% casamino acid and 50 μg/mL ampicillin to M9 medium) at 37°C for 1 ~ 2 days, during which time the glucose concentration was maintained at 1% and pH at 6.5. The culture was shaken at 750 rpm and aerated at 1 l/minute. The obtained culture was centrifuged at 8,000 rpm for 10 minutes to collect cells, and the cells were washed with a 30 mmol/l aqueous solution of sodium chloride and 30 mmol/l tris-hydrochloride buffer (pH 7.5). The washed cells were suspended in 30 mL of the above buffer and disrupted by ultrasonication (Branson Sonic Power

Company, Sonifier Cell Disruptor 200, output control 2) at 0°C for 10 minutes. The ultrasonicated cells were centrifuged at 9,000 rpm for 30 minutes to obtain cell residue.

Additionally, powerful protein denaturing agents such as carbamide, guanidine HCL, etc. were added to the cell residue and the rhIFN-γ was dissolved, following which the method of Marston, et al. [Bio/Technology, Vol. 2, p. 800 (1984)] was used to extract, purify, solubilize and regenerate the rhIFN-γ.

Reference Example 3: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor Derivative

An rhG-CSF derivative wherein threonine at position 1 was replaced with alanine, leucine at position 3 was replaced with threonine, glycine at position 4 was replaced with tyrosine, proline at position 5 was replaced with arginine and cysteine at position 17 was replaced with serine in hG-CSF having the amino acid sequence shown in SEQ ID NO: 3 was obtained by the method described in Japanese Published Examined Patent Application No. H7-96558.

Escherichia coli W3110strA carrying plasmid pCfBD28 comprising DNA encoding the above rhG-CSF derivative (Escherihica coli ECfBD28 FERM BP-1479) was cultured in LG medium (a medium prepared by dissolving 10 g of bactotrypton, 5 g of yeast extract, 5 g of sodium chloride and 1 g of glucose in 1 L of water and adjusted to pH 7.0 with NaOH) at 37°C for 18 hours. The resulting culture (5 mL) was inoculated into 100 mL of MCG medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% sodium chloride, 0.5% casamino acid, 1 mmol/l $MgSO_4$, 14 μg/mL vitamin B, pH 7.2) containing 25 μg/mL tryptophan and 50 μg/mL ampicillin. After culturing at 30°C for 4 ~ 8 hours, 10 μg/mL 3-indoleacrylic acid (hereinafter abbreviated as IAA), a tryptophan inducer, was added, followed by further culturing for 2 ~12 hours. The obtained culture was centrifuged at 8,000 rpm for 10 minutes to collect cells, and the cells were washed with a 30 mmol/l aqueous solution of sodium chloride and 30 mmol/l tris-hydrochloride buffer (pH 7.5). The washed cells were suspended in 30 mL of the above buffer and disrupted by ultrasonication (Branson Sonic Power Company, Sonifier Cell Disruptor 200, output control 2) at 0°C for 10 minutes. The ultrasonicated cells were centrifuged at 9,000 rpm for 30 minutes to obtain cell residue.

From the cell residue, the rhG-CSF derivative was extracted, purified, solubilized and regenerated in accordance with the method of Marston, et al. [Bio/Technology, Vol. 2, p. 800 (1984)].

Reference Example 4: Preparation of Recombinant Human Granulocyte-Colony Stimulating Factor

rhG-CSF having the amino acid sequence shown in SEQ ID NO: 3 was prepared according to the method described in Reference Example 3.

Industrial Field of Application

The present invention provides an ointment that contains a chemically modified physiologically active polypeptide possessing superior stability characteristics in the ointment when compared to other physiologically active polypeptides.

CLAIMS

1. An ointment comprising a chemically modified physiologically active polypeptide.

2. The ointment of Claim 1, wherein the chemically modified physiologically active polypeptide is a chemically modified physiologically active peptide comprising at least one polyethylene glycol.

3. The ointment of Claim 1 or 2, wherein the physiologically active polypeptide comprises asparaginase, glutaminase, arginase, uricase, superoxide dismutase, lactoferin, streptokinase, plasmin, adenosine deaminase, interleukin 1 through 18, interferon-α, interferon-β, interferon-γ, interferon-ω, interferon-τ, granulocyte-colony stimulating factor, erythropoietin, tumor necrosis factor, thrombopoietin, Klotho protein, leptin, fibroblast growth factor 1 through 19, midkine, calcitonin, epidermal growth factor, glucagon, insulin-like growth factor 1, osteogenic protein 1, stem cell factor, amylin, parathyroid hormone, plasminogen activator, vascular endothelial growth factor, transforming growth factor, glucagon-like peptide, natriuretic peptide, plasminogen, angiopoietin, angiostatin, endostatin, hepatocyte growth factor, antibody or fragment thereof, or fusion antibody.

4. The ointment of Claim 2 or 3, wherein the polyalkylene glycol comprises polyethylene glycol or a derivative thereof, polypropylene glycol or a derivative thereof, or polyethylene glycol polypropylene glycol polymer or a derivative thereof.

5. The ointment of any of Claims 2 through 4, wherein the polyalkylene glycol comprises a polyalkylene glycol with a molecular weight of 500 ~1,000,000.

6. A cosmetic ointment comprising a chemically modified physiologically active polypeptide of any of Claims 1 through 5.

7. An emollient ointment comprising a chemically modified physiologically active polypeptide of any of Claims 1 through 5.

8. Use of a chemically modified physiologically active peptide of any of Claims 1 through 5 for ointment production.

9. Use of a chemically modified physiologically active peptide of any of Claims 1 through 5 for cosmetic ointment production.

10. Use of a chemically modified physiologically active peptide of any of Claims 1 through 5 for emollient ointment production.

11. A method for stabilizing the physiologically active polypeptide in the ointment characterized by chemical modification of the physiologically active polypeptide with a polyalkylene glycol.

12. A method for retaining activity of the physiologically active polypeptide in the ointment characterized by chemical modification of the physiologically active polypeptide with a polyalkylene glycol.

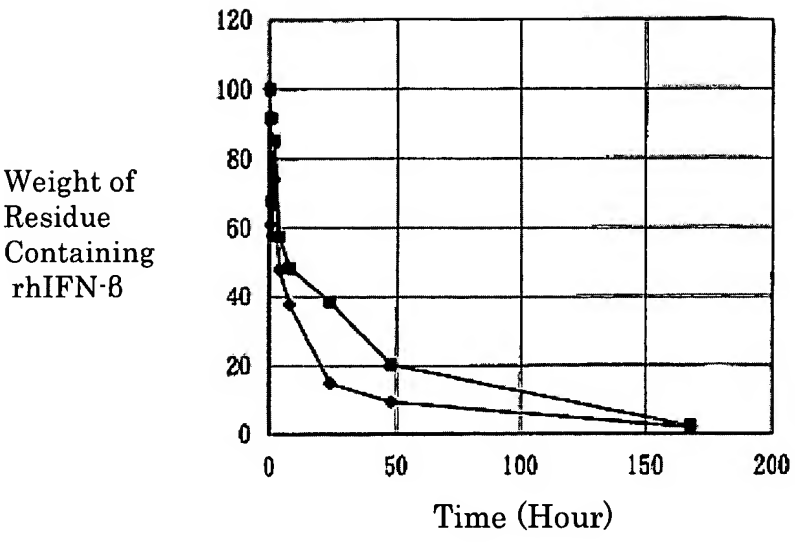
13. A composition comprising a chemically modified physiologically active polypeptide and ointment base.

14. The composition of Claim 13, wherein the chemically modified physiologically active polypeptide is a chemically modified physiologically active peptide comprising at least one polyethylene glycol.

15. The composition of Claim 13 or 14, wherein the physiologically active polypeptide comprises asparaginase, glutaminase, arginase, uricase, superoxide dismutase, lactoferin, streptokinase, plasmin, adenosine deaminase, interleukin 1 through 18, interferon-α, interferon-β, interferon-γ, interferon-ω, interferon-τ, granulocyte-colony stimulating factor, erythropoietin, tumor necrosis factor, thrombopoietin, Klotho protein, leptin, fibroblast growth factor 1 through 19, midkine, calcitonin, epidermal growth factor, glucagon, insulin-like growth factor 1, osteogenic protein 1, stem cell factor, amylin, parathyroid hormone, plasminogen activator, vascular endothelial growth factor, transforming growth factor, glucagon-like peptide, natriuretic peptide, plasminogen, angiopoietin, angiostatin, endostatin, hepatocyte growth factor, antibody or fragment thereof, or fusion antibody.

16. The composition of Claim 14 or 15 wherein the polyalkylene glycol comprises polyethylene glycol or a derivative thereof, polypropylene glycol or a derivative thereof, or polyethylene glycol polypropylene glycol polymer or a derivative thereof.

17. The composition of any of Claims 14 through 16, wherein the polyalkylene glycol comprises a polyalkylene glycol with a molecular weight of between 500 and 1,000,000.



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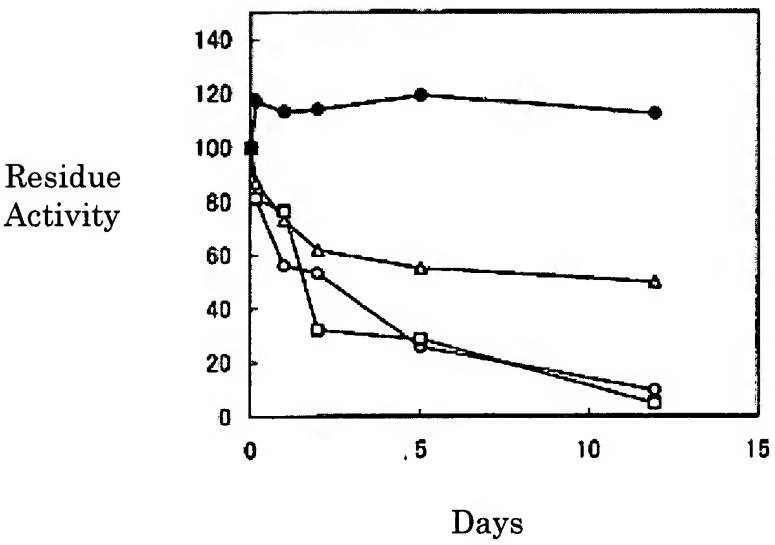

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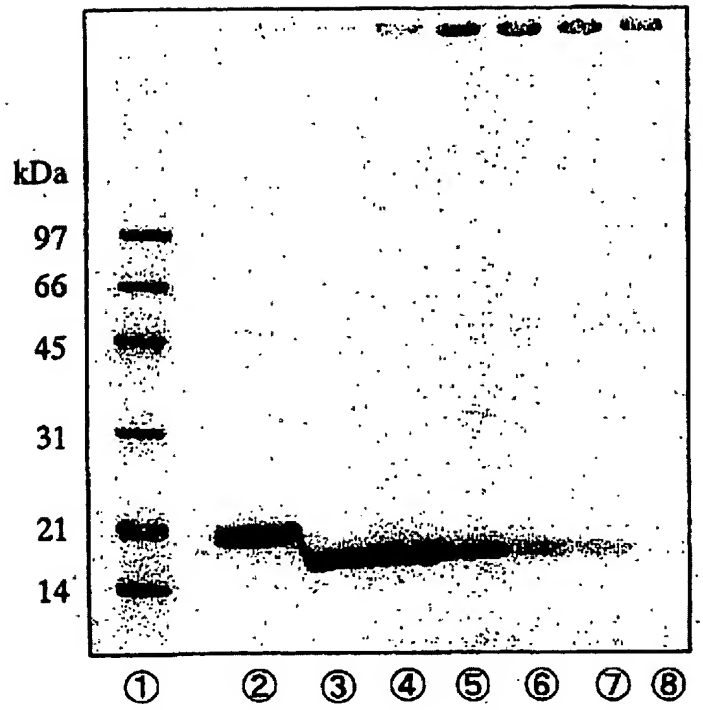

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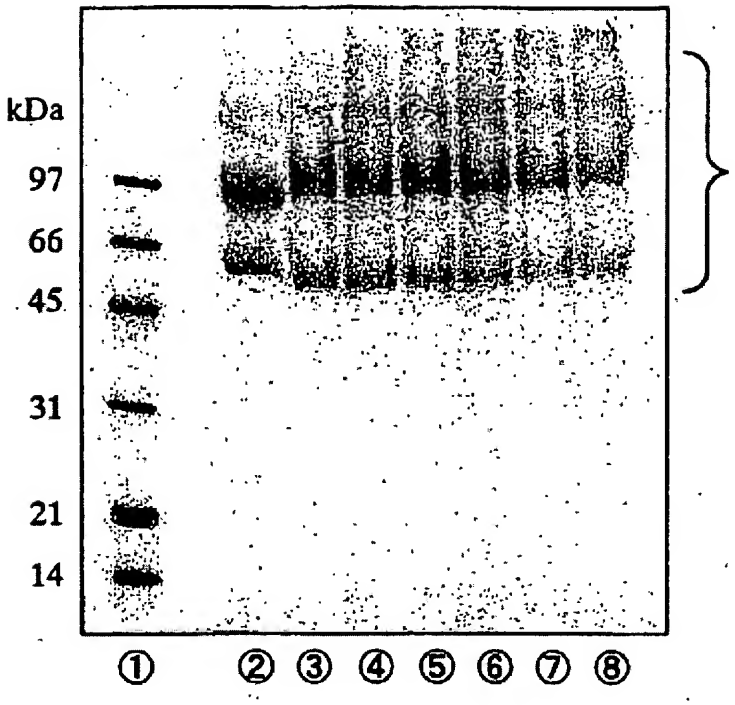
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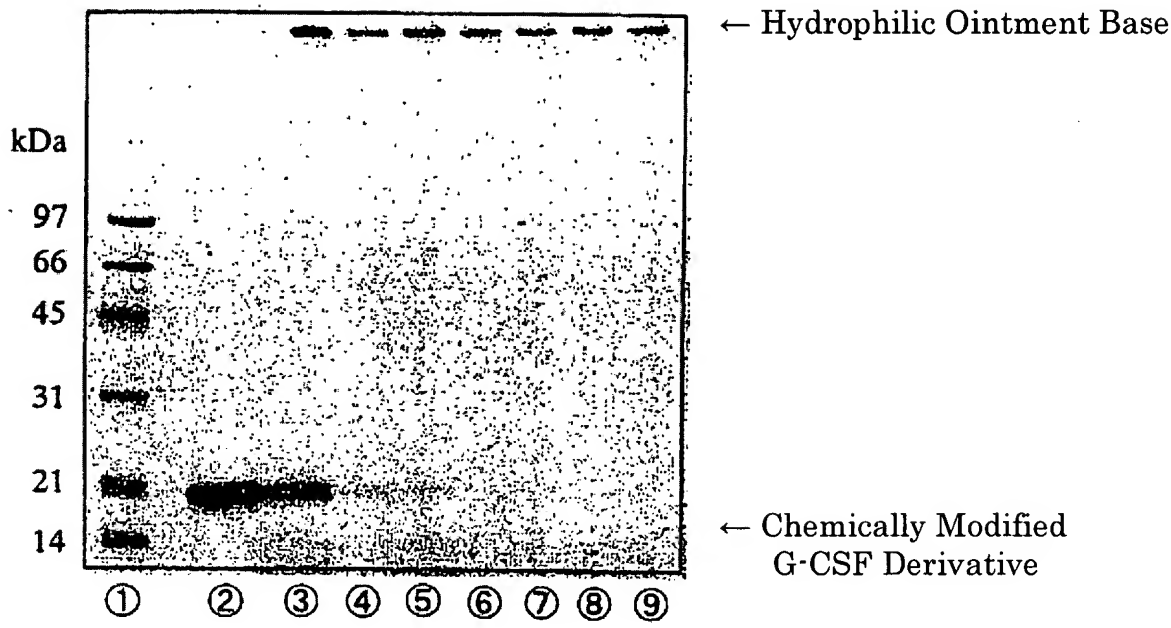
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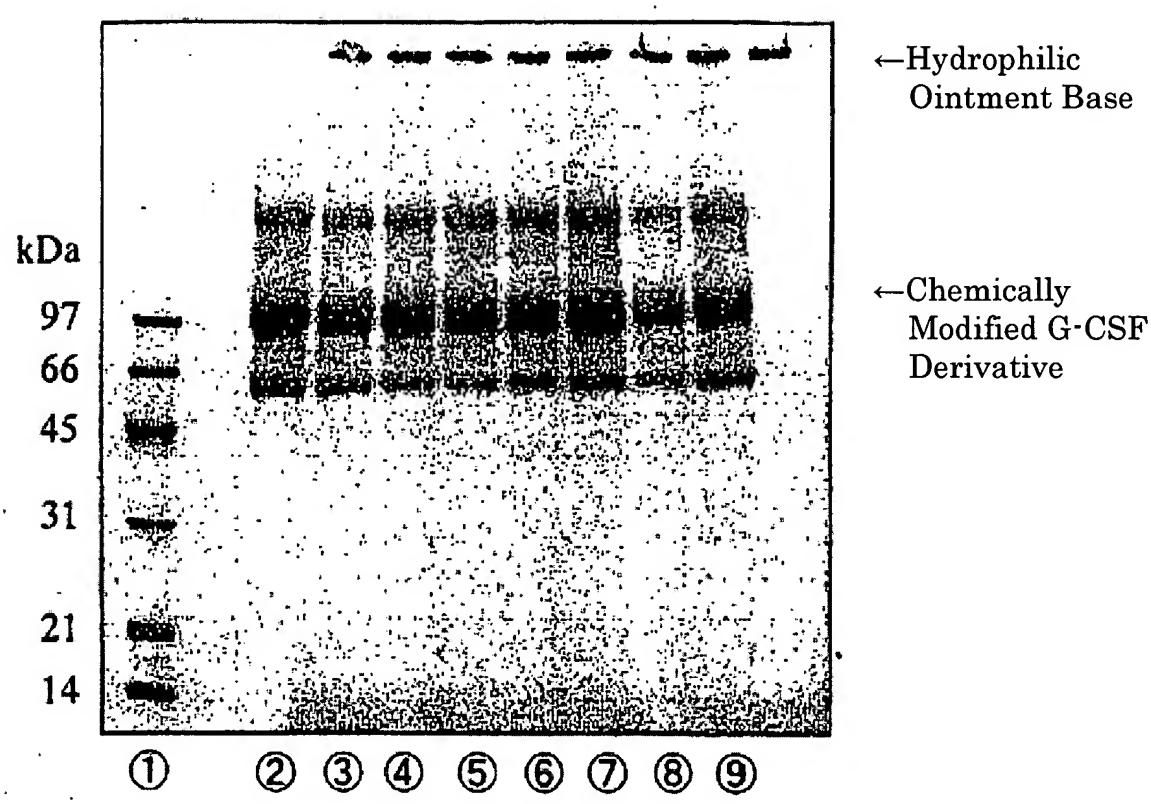
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Figure 6

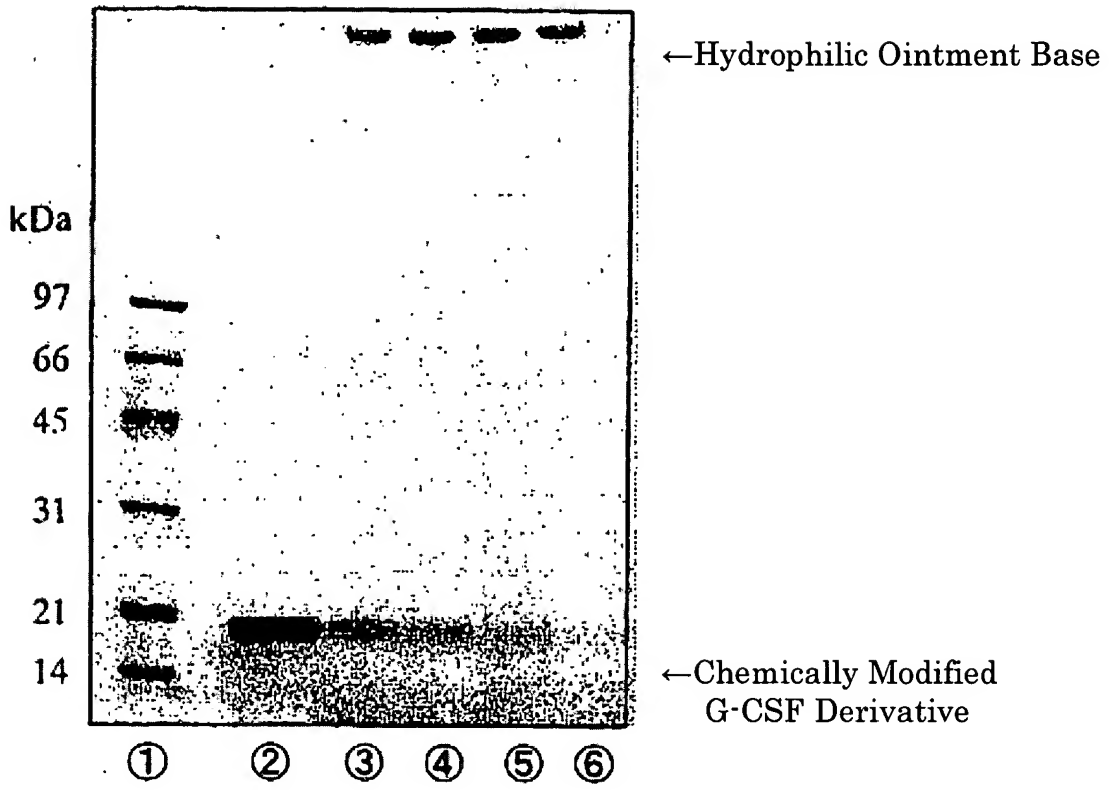
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Figure 7

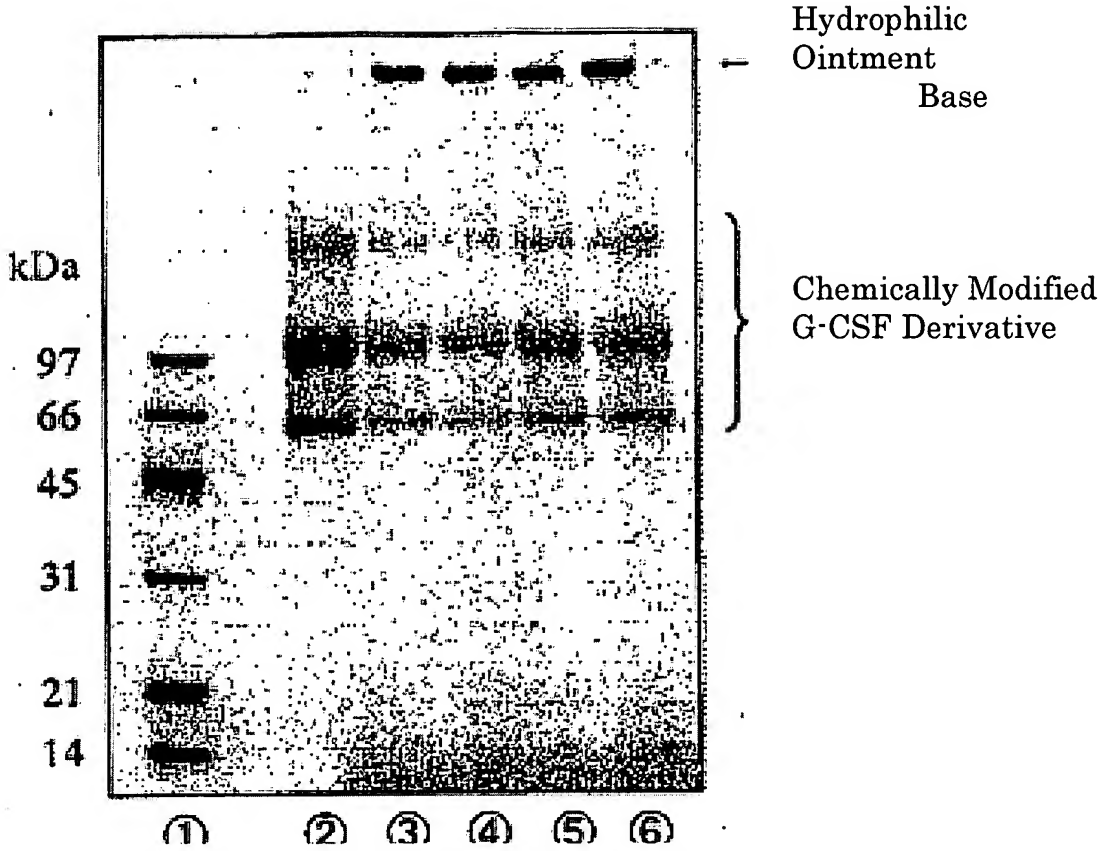
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Figure 8

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INTERNATIONAL SEARCH REPORT | International Application No. PCT/JP02/06227

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7], A61K38/00, 9/06, 7/00, 7/48, 47/30, 47/48, A61P43/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7], A61K38/00, 9/06, 7/00, 7/48, 47/30, 47/48, A61P43/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho	1926-1992	Toroku Jitsuyo Shinan Koho	1994-1996
Kokai Jitsuyo Shinan Koho	1971-1992	Jitsuyo Shinan Toroku Koho	1996-2002

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
CA (STN), MEDLINE (STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	JP 1-175999 A (Teijin Ltd.), 12 July, 1989 (12.07.89), Claims: page 9, upper right column (Family: none)	1-17
X	WO 00/033893 A1 (Johnson & Johnson Medical Ltd.), 15 June, 2000 (15.06.00), Example 11 & EP 1053029 A1 & JP 2002-531532 A	1, 3, 6-10 13, 15
X	JP 1-85934 A (Takara Shuzo Kabushiki Kaisha), 30 March, 1989 30.03.89), Page 2, upper left column, line 9; lower left column, Line 3; lower right column (Family: none)	1, 6-10, 13

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date
"T" later document published after the international priority date and not in conflict with the application but cited to understand the principle of theory underlying the invention
"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"Z" document member of the same patent family

but later than the priority date claimed	
Date of the actual completion of the international search 27 September, 2002 (27.09.02)	Date of mailing of the international search report 08 October, 2002 (08.10.02)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1998)

International Search Report

International application No. PCT/JP02/06227

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	JP 7-118165 A (Asahi Chemical Industry Co., Ltd.), 09 May, 1995 (09.05.95), Examples (Family: none)	1, 3, 8, 13, 15
Y	EP 593868 A1 (F. Hoffman-La Roche AG), 27 April, 1994 (27.04.94), Full text & JP 6-192300 A	1 - 17
Y	EP 229108 A1 (Cetus Corp.), 22 July, 1987 (22.07.87), Full text & JP 62-503171 A	1 - 17
Y	EP 210761 A1 (Takeda Chemical Industries, Ltd.), 04 February, 1987 (04.02.87), Full text & JP 62-115280 A	1 - 17

INTERNATIONAL SEARCH REPORT | International Application No. PCT/JP02/06227

A. CLASSIFICATION OF SUBJECT MATTER International Patent Classification (IPC))
Int.Cl[7], A61K38/00, 9/06, 7/00, 7/48, 47/30, 47/48, A61P43/00

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7], A61K38/00, 9/06, 7/00, 7/48, 47/30, 47/48, A61P43/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho	1926-1992	Toroku Jitsuyo Shinan Koho	1994-1996
Kokai Jitsuyo Shinan Koho	1971-1992	Jitsuyo Shinan Toroku Koho	1996-2002

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
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☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular reference
"E" earlier document by published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle of theory underlying the invention
"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"Z" document member of the same patent family

Date of the actual completion of the international search 27 September, 2002 (27.09.02)	Date of mailing of the international search report 08 October, 2002 (08.10.02)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1998)

International Search Report

International application No. PCT/JP02/06227

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	JP 1-86934 A ((illegible) 30 March, 1989 (30.03.89) 2nd page, upper left column, line 9; 2nd page, lower left column, line 3; 2nd page, lower right column (Family: none)	1, 6-10, 13
X	JP 7-118165 A (Asahi Chemical Industry Co., Ltd.), 09 May, 1995 (09.05.95), Examples (Family: none)	1, 3, 8, 13, 15
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Y	EP 229108 A1 (Cetus Corp.), 22 July, 1987 (22.07.87), Full text & JP 62-503171	1 - 17
Y	EP 210761 A1 (Takeda Chemical Industries, Ltd.), 04 February, 1987 (04.02.87), Full text & JP 62-115280 A	1 - 17